

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 February 2008 (14.02.2008)

PCT

(10) International Publication Number  
**WO 2008/019062 A2**

(51) International Patent Classification:

A61K 38/29 (2006.01)

(21) International Application Number:

PCT/US2007/017338

(22) International Filing Date: 3 August 2007 (03.08.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/835,422 4 August 2006 (04.08.2006) US

(71) Applicant (for all designated States except US): **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KRONENBERG, Henry, M.** [US/US]; 48 Hastings Road, Belmont, MA 02478 (US). **POTTS, John, T.** [US/US]; 129 Chestnut Street, Newton, MA 02465 (US). **GARDELLA, Thomas, J.** [US/US]; 1045 Greendale Avenue, Needham, MA 02492 (US).

(74) Agents: **KESSLER, Edward, J.** et al.; Sterne, Kessler, Goldstein & Fox P.L.L.c., 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

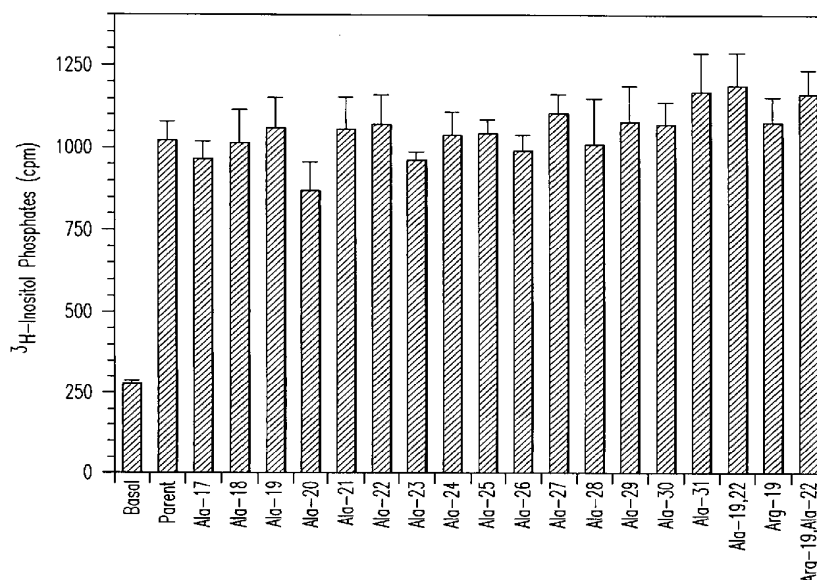
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

[Continued on next page]

(54) Title: POLYPEPTIDE DERIVATIVES OF PARATHYROID HORMONE (PTH)



(57) Abstract: The present invention is related to novel parathyroid hormone polypeptide derivatives, and to pharmaceutical composition containing the polypeptides, as well as synthetic and recombinant methods for producing the polypeptides. Also disclosed are methods for treating mammalian conditions characterized by decreases in bone mass using therapeutically effective pharmaceutical compositions containing the polypeptides of the present invention. The present invention further provides diagnostic and therapeutic methods using the polypeptide derivatives.

WO 2008/019062 A2



---

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

## POLYPEPTIDE DERIVATIVES OF PARATHYROID HORMONE (PTH)

### Statement as to Rights to Inventions Made Under Federally- Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government may have certain rights in this invention.

### BACKGROUND OF THE INVENTION

#### Field of the Invention

[0001] The present invention is related to novel parathyroid hormone (PTH) peptide derivatives, nucleic acids encoding the PTH derivatives and methods of preparing and using the PTH derivatives. The PTH derivatives may have one or more amino acid substitutions at selected positions in the polypeptide.

#### Background

##### *Parathyroid Hormone*

[0002] Parathyroid hormone (PTH), an 84 amino acid peptide, is the principal regulator of ionized blood calcium in the human body (Kronenberg, H.M., *et al.*, In *Handbook of Experimental Pharmacology*, Mundy, G.R., and Martin, T.J., (eds), pp. 185-201, Springer-Verlag, Heidelberg (1993)). Regulation of calcium concentration is necessary for the normal function of the gastrointestinal, skeletal, neurologic, neuromuscular, and cardiovascular systems. PTH synthesis and release are controlled principally by the serum calcium level; a low level stimulates and a high level suppresses both hormone

synthesis and release. PTH, in turn, maintains the serum calcium level by directly or indirectly promoting calcium entry into the blood at three sites of calcium exchange: gut, bone, and kidney. PTH contributes to net gastrointestinal absorption of calcium by favoring the renal synthesis of the active form of vitamin D. PTH promotes calcium resorption from bone indirectly by stimulating differentiation of the bone-resorbing cells, osteoclasts. It also mediates at least three main effects on the kidney: stimulation of tubular calcium reabsorption, enhancement of phosphate clearance, and promotion of an increase in the enzyme that completes synthesis of the active form of vitamin D. PTH is thought to exert these effects primarily through receptor-mediated activation of adenylate cyclase and/or phospholipase C.

[0003] Disruption of calcium homeostasis may produce many clinical disorders (*e.g.*, severe bone disease, anemia, renal impairment, ulcers, myopathy, and neuropathy) and usually results from conditions that produce an alteration in the level of parathyroid hormone. Hypercalcemia is a condition that is characterized by an elevation in the serum calcium level. It is often associated with primary hyperparathyroidism in which an excess of PTH production occurs as a result of a parathyroid gland lesion (*e.g.*, adenoma, hyperplasia, or carcinoma). Another type of hypercalcemia, humoral hypercalcemia of malignancy (HHM) is the most common paraneoplastic syndrome. It appears to result in most instances from the production by tumors (*e.g.*, squamous, renal, ovarian, or bladder carcinomas) of a class of protein hormone which shares amino acid homology with PTH. These PTH-related proteins (PTHrP) appear to mimic certain of the renal and skeletal actions of PTH and are believed to interact with the PTH receptor in these tissues.

#### *Osteoporosis*

[0004] Osteoporosis is a potentially crippling skeletal disease observed in a substantial portion of the senior adult population, in pregnant women and even

in juveniles. The term osteoporosis refers to a heterogeneous group of disorders. Clinically, osteoporosis is separated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at menopause, while osteoporosis type II is associated with advancing age. Patients with osteoporosis would benefit from new therapies designed to promote fracture repair, or from therapies designed to prevent or lessen the fractures associated with the disease.

[0005] The disease is marked by diminished bone mass, decreased bone mineral density (BMD), decreased bone strength and an increased risk of bone fracture. At present, there is no effective cure for osteoporosis, though estrogen, calcitonin and the bisphosphonates, etidronate and alendronate are used to treat the disease with varying levels of success. These agents act to decrease bone resorption. Since parathyroid hormone regulates blood calcium and the phosphate levels, and has potent anabolic (bone-forming) effects on the skeleton, in animals (Shen, V., et al., *Calcif. Tissue Int.* 50:214 220 (1992); Whitefield, J. F., et al., *Calcif. Tissue Int.* 56:227 231 (1995) and Whitfield, J. F., et al., *Calcif. Tissue Int.* 60:26 29 (1997)) and humans (Slovik, D. M., et al., *J. Bone Miner. Res.* 1:377 381 (1986); Dempster, D. W., et al., *Endocr. Rev.* 14:690 709 (1993) and Dempster, D. W., et al., *Endocr. Rev.* 15:261 (1994)) when administered intermittently, PTH, or PTH derivatives, are prime candidates for new and effective therapies for osteoporosis.

#### *PTH Derivatives*

[0006] PTH derivatives include polypeptides that have amino acid substitutions or are truncated relative to the full length molecule. Both a 14 and a 34 amino acid amino-terminal truncated form of PTH, as well as a C-terminal truncated form have been studied. Additionally, amino acid substitutions within the truncated polypeptides have also been investigated.

[0007] Synthetic PTH(1-34) exhibits full bioactivity in most cell-based assay systems, has potent anabolic effects on bone mass in animals and has been shown to reduce the risk of bone fracture in postmenopausal osteoporotic

women (Neer, R.M., *et al.*, *N.E.J.M.* 344:1434-1441 (2001); Dempster, D.W., *et al.*, *Endocr Rev* 14:690-709 (1993)). PTH acts on the PTH/PTHrP receptor (P1R), a class II G protein-coupled heptahelical receptor that couples to the adenylyl cyclase/cAMP and phospholipase C/inositol phosphate (IP) signaling pathway (Rippner, H., *et al.*, *Science* 254:1024-1026 (1991)). Deletion analysis studies have shown that the amino-terminal residues of PTH play a crucial role in stimulating the P1R to activate the cAMP and IP signaling pathways (Tregear, G.W., *et al.*, *Endocrinology* 93:1349-1353 (1973); Takasu, H., *et al.*, *Biochemistry* 38:13453-13460 (1999)). Crosslinking and receptor mutagenesis studies have indicated that residues in the amino-terminal portion of PTH interact with the extracellular loops and extracellular ends of the seven transmembrane helices, which reside within the juxtamembrane region of the receptor (Bergwitz, C., *et al.*, *J. Biol. Chem.* 271:26469-26472 (1996); Hoare, S.R.J., *et al.*, *J. Biol. Chem* 276:7741-7753 (2001); Behar, V., *et al.*, *J. Biol. Chem.* 275:9-17 (1999); Shimizu, M., *et al.*, *J. Biol. Chem.* 275:19456-19460 (2000); Luck, M.D., *et al.*, *Molecular Endocrinology* 13:670-680 (1999)).

[0008] Therefore, there exists a need in the art for new PTH derivatives that can be used to treat patients in need of treatment of diseases involving the parathyroid hormone, such as bone-related defects or diseases, methods of making and using these derivatives as well as methods of using the derivatives to treat patients with various parathyroid hormone related defects or diseases.

## BRIEF SUMMARY OF THE INVENTION

[0009] The present invention provides novel parathyroid hormone polypeptide derivatives containing amino acid substitutions at selected positions in the polypeptide. In some embodiments, the present invention provides a biologically active peptide at least 90% identical to a peptide consisting essentially of the formula: (a)  
 $X_{01}\text{ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMet}X_{02}X_{03}X_{04}\text{Gly}$

uX<sub>05</sub>X<sub>06</sub>ArgLysLysX<sub>07</sub>GlnAspVal (SEQ ID NO:1); (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-derivatives thereof; wherein: X<sub>01</sub> is Ser or Ala; X<sub>02</sub> is Glu or Arg; X<sub>03</sub> is Ala, Glu, Gln or Cha; X<sub>04</sub> is Val or Glu; X<sub>05</sub> is Trp, Ala, Glu or Cha; X<sub>06</sub> is Leu, Ala, Glu or Cha; and X<sub>07</sub> is Leu or Glu; provided that said peptide is not hPTH(1-31)NH<sub>2</sub>.

[0010] In some embodiments, the invention provides a biologically active peptide consisting essentially of the formula: (a) X<sub>01</sub>ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetX<sub>02</sub>X<sub>03</sub>X<sub>04</sub>GluX<sub>05</sub>X<sub>06</sub>ArgLysLysX<sub>07</sub>GlnAspVal (SEQ ID NO:1); (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-derivatives thereof; wherein: X<sub>01</sub> is Ser or Ala; X<sub>02</sub> is Glu or Arg; X<sub>03</sub> is Ala, Glu, Gln or Cha; X<sub>04</sub> is Val or Glu; X<sub>05</sub> is Trp, Ala, Glu or Cha; X<sub>06</sub> is Leu, Ala, Glu or Cha; and X<sub>07</sub> is Leu or Glu; provided that said peptide is not hPTH(1-31)NH<sub>2</sub>.

[0011] In some embodiments, the invention provides a peptide consisting essentially of the formula: (a) SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetGluAlaValGluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:2), SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetGluArgValGluAlaLeuArgLysLysLeuGlnAspVal (SEQ ID NO:3), SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetGluArgValGluTrpAlaArgLysLysLeuGlnAspVal (SEQ ID NO:4), SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetGluGluValGluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:5), AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgValGluGluLeuArgLysLysLeuGlnAspVal (SEQ ID NO:6), AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgValGluTrpGluArgLysLysLeuGlnAspVal (SEQ ID NO:7),

AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgVal  
 GluTrpLeuArgLysLysGluGlnAspVal (SEQ ID NO:8),  
 AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgGlu  
 GluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:9),  
 AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgChaVal  
 GluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:10),  
 AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgVal  
 GluChaLeuArgLysLysLeuGlnAspVal (SEQ ID NO:11), or  
 AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgVal  
 GluTrpChaArgLysLysLeuGlnAspVal (SEQ ID NO:12); (b) fragments  
 thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27,  
 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N-  
 or C- derivatives thereof.

[0012] In some embodiments of the present invention, the peptides can be  
 labeled with a label selected from the group consisting of radiolabel,  
 fluorescent label, bioluminescent label, and chemiluminescent label.

[0013] The present invention also provides a pharmaceutical composition  
 comprising a pharmaceutically acceptable carrier and a biologically active  
 peptide at least 90% identical to a peptide consisting essentially of the  
 formula: (a)

$X_{01}$ ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMet $X_{02}X_{03}X_{04}$ Glu  
 $X_{05}X_{06}$ ArgLysLys $X_{07}$ GlnAspVal (SEQ ID NO:1); (b) fragments thereof,  
 containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-  
 29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-  
 derivatives thereof; wherein:  $X_{01}$  is Ser or Ala;  $X_{02}$  is Glu or Arg;  $X_{03}$  is Ala,  
 Glu, Gln or Cha;  $X_{04}$  is Val or Glu;  $X_{05}$  is Trp, Ala, Glu or Cha;  $X_{06}$  is Leu,  
 Ala, Glu or Cha; and  $X_{07}$  is Leu or Glu; provided that said peptide is not  
 hPTH(1-31)NH<sub>2</sub>.

[0014] In addition, the present invention provides a nucleic acid molecule  
 consisting essentially of a polynucleotide encoding a biologically active  
 peptide at least 90% identical to a peptide consisting essentially of the



formula:

(a)

X<sub>01</sub>ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetX<sub>02</sub>X<sub>03</sub>X<sub>04</sub>GluX<sub>05</sub>X<sub>06</sub>ArgLysLysX<sub>07</sub>GlnAspVal (SEQ ID NO:1); (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-derivatives thereof; wherein: X<sub>01</sub> is Ser or Ala; X<sub>02</sub> is Glu or Arg; X<sub>03</sub> is Ala, Glu, Gln or Cha; X<sub>04</sub> is Val or Glu; X<sub>05</sub> is Trp, Ala, Glu or Cha; X<sub>06</sub> is Leu, Ala, Glu or Cha; and X<sub>07</sub> is Leu or Glu; provided that said peptide is not hPTH(1-31)NH<sub>2</sub>. The present invention also provides a recombinant vector comprising such a nucleic acid molecule.

[0015] The present invention is also directed to a recombinant DNA molecule comprising: (1) an expression control region, said region in operable linkage with (2) a polynucleotide sequence coding for a biologically active peptide at least 90% identical to a peptide consisting essentially of the formula: (a) X<sub>01</sub>ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetX<sub>02</sub>X<sub>03</sub>X<sub>04</sub>GluX<sub>05</sub>X<sub>06</sub>ArgLysLysX<sub>07</sub>GlnAspVal (SEQ ID NO:1); (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-derivatives thereof; wherein: X<sub>01</sub> is Ser or Ala; X<sub>02</sub> is Glu or Arg; X<sub>03</sub> is Ala, Glu, Gln or Cha; X<sub>04</sub> is Val or Glu; X<sub>05</sub> is Trp, Ala, Glu or Cha; X<sub>06</sub> is Leu, Ala, Glu or Cha; and X<sub>07</sub> is Leu or Glu; provided that said peptide is not hPTH(1-31)NH<sub>2</sub>. The present invention also provides a method of preparing a biologically active peptide comprising introducing into a host cell such a recombinant DNA molecule, and causing expression of said molecule. In addition, the invention provides a prokaryotic or eukaryotic host cell containing the recombinant DNA molecule. In some embodiments of the present invention, the host cell is bacterial. In some embodiments of the present invention, the expression control region of the recombinant DNA molecule includes a bacterial, viral, fungal or mammalian promoter.

[0016] The present invention also provides a method of making a recombinant vector comprising inserting into a vector a nucleic acid molecule consisting

essentially of a polynucleotide encoding a biologically active peptide at least 90% identical to a peptide consisting essentially of the formula: (a)  $X_{01}$ ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMet $X_{02}X_{03}X_{04}$ Glu $X_{05}X_{06}$ ArgLysLys $X_{07}$ GlnAspVal (SEQ ID NO:1); (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-derivatives thereof; wherein:  $X_{01}$  is Ser or Ala;  $X_{02}$  is Glu or Arg;  $X_{03}$  is Ala, Glu, Gln or Cha;  $X_{04}$  is Val or Glu;  $X_{05}$  is Trp, Ala, Glu or Cha;  $X_{06}$  is Leu, Ala, Glu or Cha; and  $X_{07}$  is Leu or Glu; provided that said peptide is not hPTH(1-31)NH<sub>2</sub>.

[0017] The present invention further provides a method for treating mammalian conditions characterized by decreases in bone mass, wherein said method comprises administering to a subject in need thereof an effective bone mass-increasing amount of a biologically active peptide and a pharmaceutically acceptable carrier, wherein said biologically active peptide is at least 90% identical to a peptide consisting essentially of the formula: (a)  $X_{01}$ ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMet $X_{02}X_{03}X_{04}$ Glu $X_{05}X_{06}$ ArgLysLys $X_{07}$ GlnAspVal (SEQ ID NO:1); (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-derivatives thereof; wherein:  $X_{01}$  is Ser or Ala;  $X_{02}$  is Glu or Arg;  $X_{03}$  is Ala, Glu, Gln or Cha;  $X_{04}$  is Val or Glu;  $X_{05}$  is Trp, Ala, Glu or Cha;  $X_{06}$  is Leu, Ala, Glu or Cha; and  $X_{07}$  is Leu or Glu; provided that said peptide is not hPTH(1-31)NH<sub>2</sub>.

[0018] In some embodiments of the present invention, the effective bone mass-increasing amount of the peptide is administered by providing to the patient DNA encoding the peptide and expressing the peptide in vivo.

[0019] The condition to be treated can be, for example, osteoporosis, including old age osteoporosis and menopausal osteoporosis.

[0020] In some embodiments of the present invention, the effective amount of the peptide for increasing bone mass is from about 0.01 µg/kg/day to about 1.0 µg/kg/day.

[0021] In some embodiments of the present invention, the method of administration of the peptide of the present invention can be, but is not limited to, parenteral, subcutaneous, or nasal insufflation.

[0022] The present invention also provides a method for determining rates of bone formation, bone resorption and/or bone remodeling comprising administering to a patient an effective amount of a biologically active peptide at least 90% identical to a peptide consisting essentially of the formula: (a) X<sub>01</sub>ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetX<sub>02</sub>X<sub>03</sub>X<sub>04</sub>GluX<sub>05</sub>X<sub>06</sub>ArgLysLysX<sub>07</sub>GlnAspVal (SEQ ID NO:1); (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30; (c) pharmaceutically acceptable salts thereof; or (d) N- or C-derivatives thereof; wherein: X<sub>01</sub> is Ser or Ala; X<sub>02</sub> is Glu or Arg; X<sub>03</sub> is Ala, Glu, Gln or Cha; X<sub>04</sub> is Val or Glu; X<sub>05</sub> is Trp, Ala, Glu or Cha; X<sub>06</sub> is Leu, Ala, Glu or Cha; and X<sub>07</sub> is Leu or Glu; provided that the peptide is not hPTH(1-31)NH<sub>2</sub>, and determining the uptake of the peptide into the bone of the patient.

[0023] Further embodiments, features, and advantages of the present inventions, as well as the structure and operation of the various embodiments of the present invention, are described in detail below with reference to the accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0024] The accompanying drawings, which are incorporated herein and form a part of the specification, illustrate one or more embodiments of the present invention and, together with the description, further serve to explain the principles of the invention and to enable a person skilled in the pertinent art to make and use the invention.

[0025] FIG. 1 shows an alanine-scan of the (17-31) domain of PTH(1-31)NH<sub>2</sub>. Residues in the (17-31) domain of PTH(1-31)NH<sub>2</sub> were individually replaced by alanine, and effects on binding to the intact PTHR (FIG. 1A and FIG. 1B) and to PTHR-delNt (FIG. 1C and FIG. 1D) were assessed by competition methods. Assays in FIG. 1A and FIG. 1B used stably transfected HKRK-B7 cells and <sup>125</sup>I-[Nle<sup>8,21</sup>,Tyr<sup>34</sup>]rPTH(1-34)NH<sub>2</sub> {<sup>125</sup>I-PTH(1-34)} tracer radioligand. Those in FIG. 1C and FIG. 1D used membranes prepared from COS-7 cells transiently transfected with PTHR-delNt and <sup>125</sup>I-[Aib<sup>13</sup>,Nle<sup>8</sup>,Gln<sup>10</sup>,Har<sup>11</sup>,Ala<sup>12</sup>,Trp<sup>14</sup>,Tyr<sup>15</sup>]PTH(1-15)NH<sub>2</sub> {<sup>125</sup>I-[Aib<sup>13</sup>,M]PTH(1-15)} tracer radioligand. To increase total binding of <sup>125</sup>I-[Aib<sup>13</sup>,M]PTH(1-15), the COS-7 cells were cotransfected with a Gα<sub>s</sub> negative-dominant mutant as described herein. Data are expressed as a percentage of the specific binding (SB) observed in the absence of unlabeled competitor ligand. The curves for the PTH(1-31) analogs shown in panels 1C and 1D were obtained by extrapolating the data to non-specific binding, determined which unlabeled [Aib<sup>13</sup>,M]PTH(1-15) at maximum concentration. Data for the parental PTH(1-31)NH<sub>2</sub> peptide (filled circles, dashed line) are shown in each graph, and that of [Aib<sup>13</sup>,M]PTH(1-15)NH<sub>2</sub> (open diamonds, solid line) are shown in panels 1C and 1D, for reference. Data are means (±s.e.m.) of three or more experiments, each performed in duplicate.

[0026] FIG. 2 shows the circular dichroism spectroscopy of alanine-substituted PTH(1-31)NH<sub>2</sub> analogs. The parental peptide PTH(1-31)NH<sub>2</sub> peptide and derivatives thereof altered by a single alanine substitution in the (17-31) domain were analyzed by CD spectroscopy. The negative deflections in mean-residue ellipticity [θ] in the 209 nm and 222 nm regions of the spectra are indicative of α-helical structure. For each peptide, the number of helical residues per peptide chain was calculated from [θ]<sub>222</sub>, and the resulting values are reported in Tables 1 and 2.

[0027] FIG. 3 shows cyclohexylalanine-scan of the (17-31) domain of [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub>. Residues in the (17-31) of [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> were individually replaced by cyclohexylalanine (Cha) and effects on

binding to the intact PTHR (FIG. 3A and FIG. 3B) and to PTHR-delNt (FIG. 3C and FIG. 3D) were assessed, as described in the description of FIG. 1. Data are means ( $\pm$ s.e.m.) of three or more experiments, each performed in duplicate.

[0028] FIG. 4 shows the effects of glutamate substitutions in the (19-28) region of [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub>. Residues in the (19-28) region of [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> were replaced by glutamic acid and the effects on binding to the intact PTHR (FIG. 4A and FIG. 4B) and to PTHR-delNt (FIG. 4C and FIG. 4D) were assessed, as described in the description of FIG. 1. Data are means ( $\pm$ s.e.m.) combined from three or more experiments, each performed in duplicate.

[0029] FIG. 5 shows a substitution analysis of arginine-20. The effects of replacing the highly conserved arginine at position 20 of PTH(1-31)NH<sub>2</sub> by various coded (Gln, Glu, Lys) or non-encoded (Nle, Cit, Orn, Apa, Gph, and PipGly) amino acids on binding to the intact PTHR (FIG. 5A and FIG. 5B) and to PTHR-delNt (FIG. 5C and FIG. 5D) were assessed by competition methods, as described in the description of FIG. 1. Data are means ( $\pm$ s.e.m.) combined from three or more experiments, each performed in duplicate.

[0030] FIG. 6 shows binding and cAMP-stimulating activities in ROS 17/2.8 cells. The parental peptide [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> and Glu- or Cha-substituted analogs thereof were evaluated for the capacity to bind to the endogenous PTHR in ROS 17/2.8 cells, and to stimulate cAMP formation in these cells (FIG. 6B). Competition binding studies were performed in intact cells using <sup>125</sup>I-PTH(1-34) as a tracer radioligand (FIG. 6A). Data are means ( $\pm$ s.e.m.) of data combined from three experiments, each performed in duplicate.

[0031] FIG. 7 shows circular dichroism spectroscopy of PTH(1-31)NH<sub>2</sub> and [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> analogs. PTH(1-31)NH<sub>2</sub> and analogs thereof substituted at positions 19 and/or 22 (FIG. 7A), or [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> and analogs thereof substituted in the (17-31) domain with cyclohexylalanine

(FIG. 7B) or glutamic acid (FIG. 7C), were analyzed by CD spectroscopy, as described in the description of FIG. 2.

- [0032] FIG. 8 shows stimulation of inositol phosphate accumulation. Intact COS-7 cells transiently transfected with the wild-type PTHR were treated with buffer alone (basal), PTH(1-31)NH<sub>2</sub> (parent), or an analog thereof containing the indicated alanine substitution, and effects on inositol phosphate accumulation were assessed. Data are means ( $\pm$  s.e.m.) of values from three experiments, each performed in duplicate.

## DETAILED DESCRIPTION OF THE INVENTION

### *Definitions*

- [0033] In order to provide a more clear understanding of the specification and claims, the following definitions are provided.
- [0034] Amino Acid Sequences: The amino acid sequences in this application use either the single letter or three letter designations for the amino acids. These designations are well known to one of skill in the art and can be found in numerous readily available references, such as for example in Cooper, G. M., *The Cell* 1997, ASM Press, Washington, D.C. or Ausubel *et al.*, *Current Protocols in Molecular Biology*, 1994. Where substitutions in a sequence may be referred to, for example, as Ser-3-->Ala, this means that the serine in the third position from the N-terminal end of the polypeptide may be replaced with another amino acid.
- [0035] Biological Activity of the Protein: This expression refers to any biological activity of the polypeptide. Examples of these activities include, but are not limited to metabolic or physiologic function of compounds of the polypeptide or derivatives thereof including similar activities or improved activities or those activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said compounds or derivatives thereof.

[0036] Cloning vector: A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, provide tetracycline resistance or ampicillin resistance.

[0037] DNA construct: As used herein, "DNA construct" should be understood to refer to a recombinant, man-made DNA, either linear or circular.

[0038] Derivative or Functional Derivative: The term "derivative" or "functional derivative" is intended to include "variants," the "derivatives," or "chemical derivatives" of the PTH molecule. A "variant" of a molecule or derivative thereof is meant to refer to a molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule or derivative thereof is meant to refer to a non-natural molecule substantially similar to either the molecules or fragments thereof.

[0039] PTH derivatives contain changes in the polypeptide relative to the native PTH polypeptide of the same size. A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same, and if both molecules possess a similar biological activity. Thus, two molecules that possess a similar activity, may be considered variants, derivatives, or analogs as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. PTH derivatives, however, need not have substantially similar biological activity to the native molecule. In some instances PTH derivatives may have substantially different activity than the native PTH. For example, a derivative may be either an antagonist or an agonist of the PTH receptor.

[0040] As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Examples of moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980) and will be apparent to those of ordinary skill in the art.

[0041] Expression vector: As used herein, an "expression vector" is a DNA construct that contains a structural gene operably linked to an expression control sequence so that the structural gene can be expressed when the expression vector is transformed into an appropriate host cell. Two DNA sequences are said to be "operably linked" if the biological activity of one region will affect the other region and also if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired sequence, or (3) interfere with the ability of the desired sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a desired DNA sequence if the promoter were capable of effecting transcription of that desired DNA sequence.

[0042] Fragment: A "fragment" of a molecule such as for example, SEQ ID NO: 1 or derivative thereof is meant to refer to any polypeptide subset of these molecules.

[0043] Fusion protein: By the term "fusion protein" is intended a fused protein comprising compounds such as for example, SEQ ID NO: 1 or derivatives thereof, either with or without a "selective cleavage site" linked at its N-terminus, which is in turn linked to an additional amino acid leader polypeptide sequence.

[0044] Gene therapy: As used herein, "gene therapy" means, *inter alia*, the ability to ameliorate, eliminate or attenuate a defect or disease by altering a



gene of interest or the product expressed by the gene of interest, by altering the genotype of the cell or organism of interest or by altering the normal pattern of gene expression of an organism. For example, this may be accomplished by replacing the gene of interest with a mutated gene, knocking out the gene of interest or inserting a different gene that produces a product that inhibits or stimulates the gene of interest or using other methods known to those of skill in the art. Generally, a recombinant polynucleotide is introduced into cells or tissues of an organism to effect a change in gene expression. The manipulation of the genetic material may be accomplished either *in vivo* or *ex vivo*. The above examples are not meant to limit the different ways in which the gene therapy may be effected. Any techniques known to those of skill in the art of gene therapy may be used with the claimed invention.

**[0045]** Host Animal: The term transgenic animals refers to those animals whose germ and somatic cells contain a DNA construct of the invention. Such transgenic animals are in general vertebrates. Preferred host animals are mammals such as non-human primates, mice, sheep, pigs, cattle, goats, guinea pigs, rodents, e.g. rats, and the like. The term "host animal" also includes animals in all stages of development, including embryonic and fetal stages.

**[0046]** % Identity: Whether any two polypeptides or polynucleotides are for example, at least 90% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 (1988). Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine identity.

**[0047]** The terms homology and identity are often used interchangeably. In this regard, percent homology or identity may be determined by methods known to those of skill in the art. For example, by comparing sequence information using a GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443 (1970), as revised by Smith and Waterman (*Adv. Appl. Math.*

2:482 (1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences.

[0048] In general, sequences are aligned so that the highest order match is obtained. "Identity" per se has an art-recognized meaning and can be calculated using published techniques. (See, e.g.: Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H. & Lipton, D., *SIAM J Applied Math* 48:1073 (1988)). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H. & Lipton, D., *SIAM J Applied Math* 48:1073 (1988). Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., *Nucleic Acids Research* 12(i):387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S. F., et al., *J Molec Biol* 215:403 (1990)).

[0049] Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. More specifically, a test polypeptide may be defined as any polypeptide that is 90% or more identical to a reference polypeptide. As used herein, the term at least "90% identical to" refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the

fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids, that no more than 10% (i.e., 10 out of 100) amino acids in the test polypeptides differ from that of the reference polypeptides. Such differences may be represented as point mutations randomly distributed over the entire length of the amino acid sequence of the invention or they may be clustered in one or more locations of varying length up to the maximum allowable amino acid difference (approximately 90% identity). Differences are defined as amino acid substitutions, or deletions.

**[0050]** Isolated: A term meaning altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of compounds of for example SEQ ID NO:1 and derivatives thereof can be substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31 40 (1988). The terms isolated and purified are sometimes used interchangeably.

**[0051]** By "isolated" is meant that the DNA is free of the coding sequences of those genes that, in the naturally-occurring genome of the organism (if any) from which the DNA of the invention is derived, immediately flank the gene encoding the DNA of the invention. The isolated DNA may be single-stranded or double-stranded, and may be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It may be identical to a native DNA sequence encoding compounds of for example, SEQ ID NO:1 and derivatives thereof, or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides. Single-stranded DNAs of the invention are generally at least 8 nucleotides long, (preferably at least 18 nucleotides long, and more

preferably at least 30 nucleotides long) ranging up to full length of the DNA molecule encoding compounds of SEQ ID NO:1 and derivatives thereof; they preferably are detectably labeled for use as hybridization probes, and may be antisense.

**[0052]** Isolated or purified as it refers to preparations made from biological cells or hosts should be understood to mean any cell extract containing the indicated DNA or protein including a crude extract of the DNA or protein of interest. For example, in the case of a protein, a purified preparation can be obtained following an individual technique or a series of preparative or biochemical techniques and the DNA or protein of interest can be present at various degrees of purity in these preparations. The procedures may include for example, but are not limited to, ammonium sulfate fractionation, gel filtration, ion exchange chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

**[0053]** A preparation of DNA or protein that is "pure" or "isolated" should be understood to mean a preparation free from naturally occurring materials with which such DNA or protein is normally associated in nature. "Essentially pure" should be understood to mean a "highly" purified preparation that contains at least 95% of the DNA or protein of interest.

**[0054]** A cell extract that contains the DNA or protein of interest should be understood to mean a homogenate preparation or cell-free preparation obtained from cells that express the protein or contain the DNA of interest. The term "cell extract" is intended to include culture media, especially spent culture media from which the cells have been removed.

**[0055]** Leader Sequence: By the term "leader sequence" is intended a polynucleotide sequence linked to compounds of for example, SEQ ID NO: 1, and expressed in host cells as a fusion protein fused to the selective cleavage site and compounds of SEQ ID NO: 1. The term "leader polypeptide" describes the expressed form of the "leader sequence" as obtained in the fusion protein.

[0056] The fusion protein, which is often insoluble and found in inclusion bodies when it is overexpressed, is purified from other bacterial protein by methods well known in the art. In a preferred embodiment, the insoluble fusion protein is centrifuged and washed after cell lysis, and resolubilized with guanidine-HCl. It can remain soluble after removal of the denaturant by dialysis. (For purification of refractile proteins, see Jones, U.S. Pat. No. 4,512,922; Olson, U.S. Pat. No. 4,518,526; and Builder *et al.*, U.S. Pat. Nos. 4,511,502 and 4,620,948).

[0057] The recombinantly produced compounds of for example, SEQ ID NO: 1 or derivatives thereof can be purified to be substantially free of natural contaminants from the solubilized fusion protein through the use of any of a variety of methodologies. As used herein, a compound is said to be "substantially free of natural contaminants" if it has been substantially purified from materials with which it is found following expression in bacterial or eukaryotic host cells. Compounds of SEQ ID NO: 1 or derivatives thereof may be purified through application of standard chromatographic separation technology.

[0058] Alternatively, the peptide may be purified using immuno-affinity chromatography (Rotman, A. *et al.*, *Biochim. Biophys. Acta* 641:114 121 (1981); Sairam, M. R. J, *Chromatog* 215:143 152 (1981); Nielsen, L. S. *et al.*, *Biochemistry* 21:6410 6415 (1982); Vockley, J. *et al.*, *Biochem. J.* 217:535 542 (1984); Paucha, E. *et al.*, *J. Virol.* 51:670 681 (1984); and Chong, P. *et al.*, *J. Virol. Meth.* 10:261 268 (1985)).

[0059] After partial or substantial purification, the fusion protein is treated enzymatically with the enzyme corresponding to the cleavage site. Alternatively, the fusion protein in its more impure state, even in refractile form, can be treated with the enzyme. If needed, the resulting mature compounds of for example, SEQ ID NO: 1 or derivatives thereof, can be further purified. Conditions for enzymatic treatment are known to those of skill in the art.

**[0060]** Operably Linked: Two DNA sequences (such as a promoter region sequence and a sequence encoding a PTH derivative) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired sequence, or (3) interfere with the ability of the desired sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a desired DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

**[0061]** Polynucleotide: This term generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications have been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

**[0062]** Polypeptide: Polypeptide and peptide are used interchangeably. The term polypeptide refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly

referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids and include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in the research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications.

[0063] Polypeptides may be branched and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translational modifications or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, *Proteins-Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., *Posttranslational Protein Modifications: Perspectives and Prospects*, pgs. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al, "Analysis for protein modifications and nonprotein

cofactors", *Methods in Enzymol.* 182:626-646 (1990) and Rattan *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci* 663:48-62 (1992).

**[0064]** Promoter: A DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Examples of promoters include the CMV promoter (Invitrogen, San Diego, Calif.), the SV40, MMTV, and hMTIIa promoters (U.S. Pat. No. 5,457,034), the HSV-1 4/5 promoter (U.S. Pat. No. 5,501,979), and the early intermediate HCMV promoter (WO92/17581). Also, tissue-specific enhancer elements may be employed. Additionally, such promoters may include tissue and cell-specific promoters of an organism.

**[0065]** Recombinant Host: According to the invention, a recombinant host may be any prokaryotic or eukaryotic host cell which contains the desired cloned genes on an expression vector or cloning vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the desired gene(s) in the chromosome or genome of that organism. For examples of such hosts, see Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). Preferred recombinant hosts are eukaryotic cells transformed with the DNA construct of the invention. More specifically, mammalian cells are preferred.

**[0066]** Selective cleavage site: The term "selective cleavage site" refers to an amino acid residue or residues which can be selectively cleaved with either chemicals or enzymes in a predictable manner. A selective enzyme cleavage site is an amino acid or a peptide sequence which is recognized and hydrolyzed by a proteolytic enzyme. Examples of such sites include, without limitation, trypsin or chymotrypsin cleavage sites.



[0067] Stringent Hybridization. As used herein "stringent hybridization" conditions should be understood to be those conditions normally used by one of skill in the art to establish at least a 95% homology between complementary pieces of DNA or DNA and RNA.

[0068] There are only three requirements for hybridization to a denatured strand of DNA to occur. (1) There must be complementary single strands in the sample. (2) The ionic strength of the solution of single-stranded DNA must be fairly high so that the bases can approach one another; operationally, this means greater than 0.2M. (3) The DNA concentration must be high enough for intermolecular collisions to occur at a reasonable frequency. The third condition only affects the rate, not whether renaturation/hybridization will occur.

[0069] Conditions routinely used by those of skill in the art are set out in readily available procedure texts, e.g., Ausubel, F. et al., *Current Protocols in Molecular Biology*, Vol. I, Chap. 2.10, John Wiley & Sons, Publishers (1994) or Sambrook *et al.*, *Molecular Cloning*, Cold Spring Harbor (1989), the entire documents incorporated herein by reference. As would be known by one of skill in the art, the ultimate hybridization stringency reflects both the actual hybridization conditions as well as the washing conditions following the hybridization, and one of skill in the art would know the appropriate manner in which to change these conditions to obtain a desired result.

[0070] For example, a prehybridization solution should contain sufficient salt and nonspecific DNA to allow for hybridization to non-specific sites on the solid matrix, at the desired temperature and in the desired prehybridization time. For example, for stringent hybridization, such prehybridization solution could contain 6x sodium chloride/sodium citrate (1x SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5x Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg per ml of herring sperm DNA. An appropriate stringent hybridization mixture might then contain 6x SSC, 1x Denhardt's solution, 100 µg per ml of yeast tRNA and 0.05% sodium pyrophosphate.

- [0071] Alternative conditions for DNA--DNA analysis could entail the following: 1) prehybridization at room temperature and hybridization at 68°C; 2) washing with 0.2 x SSC/0.1% SDS at room temperature; 3) as desired, additional washes at 0.2 x SSC/0.1% SDS at 42°C (moderate-stringency wash); or 4) as desired, additional washes at 0.1 x SSC/0.1% SDS at 68°C (high stringency).
- [0072] Known hybridization mixtures, e.g., that of Church and Gilbert, *Proc. Natl. Acad. Sci. USA* 81:1991 1995 (1984), comprising the following composition may also be used: 1% crystalline grade bovine serum albumin/1 mM EDTA/0.5M NaHPO<sub>4</sub>, pH 7.2/7% SDS. Additionally, alternative but similar reaction conditions can also be found in Sambrook *et al.*, *Molecular Cloning*, Cold Spring Harbor (1989). Formamide may also be included in prehybridization/hybridization solutions as desired. The invention may include DNA sequences that stringently hybridize to nucleic acid sequences encoding PTH derivatives.
- [0073] Transgenic: As used herein, a "transgenic" organism is an organism containing a transgene, wherein the transgene was introduced into the organism or an ancestor of the organism at a prenatal stage, e.g., an embryonic stage. The transgene results in a defined change to its germ line, wherein the change is not ordinarily found in wild-type organisms. This change can be passed on to the organism's progeny and therefore the progeny are also transgenic animals. The change to the organism's germ line can be an insertion, a substitution, or a deletion in the gene of interest. Non-human animals are organisms into which transgenes may be introduced by techniques known in the art, such animals include but are not limited to mice, goats, sheep, pigs, cows and other domestic farm animals. Methods for generating transgenic animals have become convention in the art and are described, for example, in Hogan B. *et al.*, "A Laboratory Manual, Cold Spring Harbor, N.Y. (1986) or U.S. Pat. No. 5,922,927 or 5,917,123. A transgenic animal that carries one transgene can be further bred to another transgenic animal carrying

a second transgene to create a "double transgenic" animal carrying two transgenes.

*Compounds of the Invention*

[0074] Frequently in this section, reference is made to the polypeptide of SEQ ID NO:1. This is merely illustrative and should not be meant to imply that this is limiting in any way relative to the other polypeptide sequences of the invention. As protein products, compounds of the invention are amenable to production by the techniques of solution- or solid-phase peptide synthesis or recombinant biology.

[0075] The solid phase peptide synthesis technique, in particular, has been successfully applied in the production of human PTH and can be used for the production of compounds of SEQ ID NO: 1, derivatives thereof (for guidance, see Kimura *et al.*, supra, and see Fairwell *et al.*, *Biochem.* 22:2691 (1983)). Success with producing human PTH on a relatively large scale has been reported by Goud *et al.*, in *J. Bone Min. Res.* 6:781 (1991), incorporated herein by reference. The synthetic peptide synthesis approach generally entails the use of automated synthesizers and appropriate resin as solid phase, to which is attached the C-terminal amino acid of the desired compounds of SEQ ID NO: 1 or derivatives thereof. Extension of the peptide in the N-terminal direction is then achieved by successively coupling a suitably protected form of the next desired amino acid, using either FMOC- or BOC-based chemical protocols typically, until synthesis is complete. Protecting groups are then cleaved from the peptide, usually simultaneously with cleavage of peptide from the resin, and the peptide is then isolated and purified using conventional techniques, such as by reversed phase HPLC using acetonitrile as solvent and tri-fluoroacetic acid as ion-pairing agent. Such procedures are generally described in numerous publications and reference may be made, for example, to Stewart and Young, "Solid Phase Peptide Synthesis," 2nd Edition, Pierce Chemical Company, Rockford, Ill. (1984). It will be appreciated that the peptide synthesis approach is required for production of such as for example,

SEQ ID NO: 1 and derivatives thereof which incorporate amino acids that are not genetically encoded, such as cyclohexylalanine (Cha).

[0076] In one aspect of the invention, any amino-acid substitutions at positions 17-31 of PTH, particularly those amino acid substitutions at amino acid positions 19, 20, 21, 23, 24, 25, 26, 27, 28, and/or 31, and more particularly those amino acid substitutions at amino acid positions 20, 21, 23, 24, and/or 28, which do not destroy the biological activity of the PTH polypeptide to antagonize or agonize the PTH-1/PTH-2 receptor (as determined by assays known to the skilled artisan and discussed herein), are also included within the scope of the present invention.

[0077] The synthetic analog of bovine PTH, PTH(3-34) has been recognized as a potent PTH antagonist in vitro. Variants of PTH lacking N-terminal amino acids 1-2 and 1-7, were shown to be devoid of agonist activity and capable of antagonist activity (Born, W. *et al.*, *Endocrinol.* 23:1848-1853 (1988)). Preferred potential antagonist variants of SEQ ID NO: 1 of this invention are variants truncated at the N-terminus.

[0078] In accordance with another aspect of the present invention, substituents may be attached to the free amine of the N-terminal amino acid of compounds such as, for example, SEQ ID NO: 1 or derivatives thereof by standard methods known in the art. For example, alkyl groups, e.g., C<sub>1-12</sub> alkyl, may be attached using reductive alkylation. Hydroxyalkyl groups, e.g. C<sub>1-12</sub> hydroxyalkyl, may also be attached using reductive alkylation wherein the free hydroxy group is protected with a t-butyl ester. Acyl groups, e.g., COE<sub>1</sub>, may be attached by coupling the free acid, e.g., E<sub>1</sub>COOH, to the free amino of the N-terminal amino acid. Additionally, possible chemical modifications of the C-terminal end of the polypeptide are encompassed within the scope of the invention. These modifications may modify binding affinity to the receptor.

[0079] Also contemplated within the scope of this invention are those compounds such as for example, SEQ ID NO:1 and derivatives thereof that alter secondary or tertiary structure, or stability of compounds such as SEQ ID NO: 1 or derivatives thereof which still retain biological activity. Such

derivatives might be achieved through lactam cyclization, disulfide bonds, or other means known to a person of ordinary skill in the art.

[0080] The peptides of the invention may also be labeled. The labeling molecule may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorescent label, a chemiluminescent label, a bioluminescent label, a paramagnetic ion, a radioisotope including other nuclear tags and a direct visual label.

[0081] In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

[0082] Routinely used radiolabels include radiolabels  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$  and  $^{32}\text{P}$ . Techniques to incorporate radioisotopes are well known in the art. (See, Current Protocols in Protein Science, Coligan *et al.* eds., John Wiley Sons Inc. 2002)

[0083] A large number of enzymes suitable for use as labeling molecules is disclosed in U.S. Pat. Nos. 4,366,241, 4,843,000, and 4,849,338. Suitable enzymes useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase,  $\beta$ -galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzymes may be used alone or in combination with a second enzyme that is in solution.

[0084] Suitable fluorescent labels (or fluorochromes) include, but are not limited to, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), R-Phycoerythrin (RPE), and Texas Red. Other exemplary fluorochromes include those discussed by Dower et al., International Publication No. WO 93/06121. Reference also may be made to the fluorochromes described in U.S. Pat. Nos. 5,573,909 (Singer et al), U.S. Pat. No. 5,326,692 (Brinkley et al). Alternatively, reference may be made to the fluorochromes described in U.S. Pat. Nos. 5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663, 5,453,517, 5,459,276, 5,516,864, 5,648,270 and 5,723,218.

*Vectors, Host Cells, and Recombinant Expressions*

- [0085] The present invention also relates to vectors that comprise a polynucleotide of the present invention, and host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of present invention.
- [0086] For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, (such as Davis *et al.*, *Basic Methods in Molecular Biology* (1986) and Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.
- [0087] Representative examples of appropriate hosts include bacterial cells, such as *Streptococci*, *Staphylococci*, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera Sf9* cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.
- [0088] A great variety of expression systems can be used. Such systems include, among others, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses, and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions

that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (supra).

[0089] RNA vectors may also be utilized for the expression of the nucleic acids encoding compounds of the invention or derivatives thereof disclosed in this invention. These vectors are based on positive or negative strand RNA viruses that naturally replicate in a wide variety of eukaryotic cells (Bredenbeek, P. J. & Rice, C. M., *Virology* 3: 297-310, 1992). Unlike retroviruses, these viruses lack an intermediate DNA life-cycle phase, existing entirely in RNA form. For example, alpha viruses are used as expression vectors for foreign proteins because they can be utilized in a broad range of host cells and provide a high level of expression; examples of viruses of this type include the Sindbis virus and Semliki Forest virus (Schlesinger, S., *TIBTECH* 11:18-22, 1993; Frolov, I., et al., *Proc. Natl. Acad. Sci. (USA)* 93: 11371-11377, 1996). As exemplified by Invitrogen's Sinbis expression system, the investigator may conveniently maintain the recombinant molecule in DNA form (pSinrep5 plasmid) in the laboratory, but propagation in RNA form is feasible as well. In the host cell used for expression, the vector containing the gene of interest exists completely in RNA form and may be continuously propagated in that state if desired.

[0090] For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment appropriate secretion signals may be incorporated into the desired polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

[0091] The expression of a DNA sequence requires that the DNA sequence be "operably linked" to DNA sequences which contain transcriptional and translational regulatory information. An operable linkage is a linkage in which

the control or regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the "control regions" needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotic cells, contains both the promoter (which directs the initiation of RNA transcription) as well as DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Regulatory regions in eukaryotic cells will in general include a promoter region sufficient to direct the initiation of RNA synthesis.

[0092] The joining of various DNA fragments, to produce the expression vectors of this invention is performed in accordance with conventional techniques, employing blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkali and phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. In the case of a fusion protein, the genetic construct encodes an inducible promoter which is operably linked to the 5' gene sequence of the fusion protein to allow efficient expression of the fusion protein.

[0093] To express compounds of the invention or a derivative thereof in a prokaryotic cell (such as, for example, *E. coli*, *B. subtilis*, *Pseudomonas*, *Streptomyces*, etc.), it is necessary to operably link, for example, the SEQ ID NO: 1-encoding DNA sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Examples of constitutive promoters include the int promoter of bacteriophage  $\lambda$ , the bla promoter of the  $\beta$ -lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325, etc. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage  $\lambda$ , (PL and PR), the trp, recA, lacZ, lacI, and gal promoters of *E. coli*, the  $\alpha$ -amylase (Ulmanen, I. et al., *J. Bacteriol.* 162:176-182 (1985)), and the  $\sigma$ -28-specific promoters of *B. subtilis* (Gilman, M. Z. et al., *Gene* 32:11-20 (1984)), the promoters of the



bacteriophages of *Bacillus* (Gryczan, T. J., In: *The Molecular Biology of the Bacilli*, Academic Press, Inc., NY (1982)), and *Streptomyces* promoters (Ward, J. M. *et al.*, *Mol. Gen. Genet.* 203:468-478 (1986)). Prokaryotic promoters are reviewed by Glick, B. R., *J. Ind. Microbiol.* 1:277-282 (1987); Cenatiempo, Y., *Biochimie* 68:505-516 (1986)); and Gottesman, S., *Ann. Rev. Genet.* 18:415-442 (1984)).

[0094] The preferred prokaryotic promoter for this invention is the *E. coli* *trp* promoter, which is inducible with indole acrylic acid.

[0095] If expression is desired in a eukaryotic cell, such as yeast, fungi, mammalian cells, or plant cells, then it is necessary to employ a promoter capable of directing transcription in such a eukaryotic host. Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, D. *et al.*, *J. Mol. Appl. Gen.* 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell* 31:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature (London)* 290:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S. A., *et al.*, *Proc. Natl. Acad. Sci. (USA)* 79:6971-6975 (1982); Silver, P. A., *et al.*, *Proc. Natl. Acad. Sci. (USA)* 81:5951-5955 (1984)).

[0096] Preferably, the introduced gene sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

[0097] Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColE1, pSC101, pACYC 184,  $\pi$ VX. Such plasmids are, for example, disclosed by Maniatis, T., *et al.*, In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor

Press, Cold Spring Harbor, N.Y. (1982)). Preferred plasmid expression vectors include the pGFP-1 plasmid described in Gardella *et al.*, *J. Biol. Chem.* 265:15854-15859 (1989), or a modified plasmid based upon one of the pET vectors described by Studier and Dunn, *Methods in Enzymology* 185: 60-89 (1990). *Bacillus* plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. In: *The Molecular Biology of the Bacilli*, Academic Press, NY pp. 307-329 (1982). Suitable *Streptomyces* plasmids include pIJIOI (Kendall, K. J. *et al.*, *J. Bacteriol.* 169:4177-4183 (1987)), and *streptomyces* bacteriophages such as  $\Phi$ C31 (Chater, K.F. *et al.*, In: *Sixth International Symposium on Actinomycetales Biology*, Akademiai Kiado, Budapest, Hungary, pp. 45-54 (1986)). *Pseudomonas* plasmids are reviewed by John, J. F. *et al.*, *Rev. Infect. Dis.* 8:693-704 (1986)), and Izaki, K., *Jon. J. Bacteriol.* 33:729-742 (1978)).

[0098] Preferred eukaryotic expression vectors include, without limitation, BPV, vaccinia, 2-micron circle etc. Such expression vectors are well known in the art (Botstein, D., *et al.*, *Miami Wntr. Symp.* 19:265-274 (1982); Broach, J. R., In: *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp. 445-470 (1981); Broach, J. R., *Cell* 28:203-204 (1982); Bollon, D. P., *et al.*, *J. Clin. Hematol. Oncol.* 10:39-48 (1980); Maniatis, T., In: *Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Expression*, Academic Press, NY, pp. 563-608 (1980)).

[0099] In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate cellular sources. Interest, however, has been greater with cells from vertebrate sources. Examples of useful vertebrate host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, WI38, BHK, COS-7, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of or upstream to the gene to

be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

[00100] For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, Simian Virus 40 (SV40) and cytomegalovirus. The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers *et al.*, *Nature* 273:113 (1978)).

[00101] An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV) source or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

[00102] If cells without formidable cell membrane barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Erb, *Virology* 52:546 (1978). However, other methods for introducing DNA into cells, such as by nuclear injection or by protoplast fusion may also be used. In the case of gene therapy, the direct naked plasmid or viral DNA injection method, with or without transfection-facilitating agents such as, without limitation, liposomes, provides an alternative approach to the current methods of in vivo or in vitro transfection of mammalian cells. If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment, using calcium chloride as described in Cohen *et al.*, *Proc. Natl. Acad. Sci. USA* 69:2110 (1972).

#### *Gene Therapy*

[00103] A patient (human or non-human) suffering from symptoms of a disease such as osteoporosis or other diseases requiring PTH may be treated by gene therapy. By undertaking this approach, there should be an attenuation of the

disease symptoms. Gene therapy has proven effective or has been considered to have promise in the treatment of certain forms of human hemophilia (Bontempo, F. A., *et al.*, *Blood* 69:1721-1724 (1987); Palmer, T. D., *et al.*, *Blood* 73:438-445 (1989); Axelrod, J. H., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:5173-5177 (1990); Armentano, D., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:6141-6145 (1990)), as well as in the treatment of other mammalian diseases such as cystic fibrosis (Drumm, M. L., *et al.*, *Cell* 62:1227-1233 (1990); Gregory, R. J., *et al.*, *Nature* 347:358-363 (1990); Rich, D. P., *et al.*, *Nature* 347:358-363 (1990)), Gaucher disease (Sorge, J., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:906-909 (1987); Fink, J. K., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2334-2338 (1990)), muscular dystrophy (Partridge, T. A., *et al.*, *Nature* 337:176-179 (1989); Law, P. K., *et al.*, *Lancet* 336:114-115 (1990); Morgan, J. E., *et al.*, *J. Cell Biol.* 111:2437-2449 (1990)), and metastatic melanoma (Rosenberg, S. A., *et al.*, *Science* 233:1318-1321 (1986); Rosenberg, S. A., *et al.*, *N. Eng. J. Med.* 319:1676-1680 (1988); Rosenberg, S. A., *et al.*, *N. Eng. J. Med.* 323:570-578 (1990)). More recently, gene therapy has been shown to provide anticancer or antitumor activity in patients with prostate cancer (Herman, J. R. *et al.*, *Hum. Gene Ther.* 10:1239-1249 (1999)) and metastatic melanoma (Nemunaitis, J. *et al.*, *Hum. Gene Ther.* 20:1289-1298 (1999)). Additionally, several patents have issued to methods of gene therapy. For example, U.S. Pat. Nos. 5,836,905, 5,741,486, 5,871,486 and 5,656,465.

**[00104]** In a preferred approach, a polynucleotide having the nucleotide sequence for the PTH polypeptide derivative may be incorporated into a vector suitable for introducing the nucleic acid molecule into cells of the mammal to be treated, to form a transfection vector.

**[00105]** A variety of vectors have been developed for gene delivery and possible gene therapy. Suitable vectors for this purpose include retroviruses, adenoviruses and adeno associated viruses (AAV). Alternatively, the nucleic acid molecules of the invention may be complexed into a molecular conjugate with a virus (e.g., an adenovirus) or with viral components (e.g., viral capsid

proteins). The vectors derive from herpes simplex virus type 1 (HSV-1), adenovirus, adeno-associated virus (AAV) and retrovirus constructs (for review see Friedmann, T., *Trends Genet* 10:210-214 (1994); Jolly, D., *Cancer Gene Therapy* 1 (1994); Mulligan, R. C., *Science* 260:926-932 (1993); Smith, F. *et al.*, *Rest. Neurol. Neurosci.* 8:21-34 (1995)). Vectors based on HSV-1, including both recombinant virus vectors and amplicon vectors, as well as adenovirus vectors can assume an extrachromosomal state in the cell nucleus and mediate limited, long term gene expression in postmitotic cells, but not in mitotic cells. HSV-1 amplicon vectors can be grown to relatively high titers ( $10^7$  transducing units/ml) and have the capacity to accommodate large fragments of foreign DNA (at least 15 kb, with 10 concatemeric copies per virion). AAV vectors (rAAV), available in comparable titers to amplicon vectors, can deliver genes (<4.5 kb) to postmitotic, as well as mitotic cells in combination with adenovirus or herpes virus as helper virus. Long term transgene expression is achieved by replication and formation of "episomal" elements and/or through integration into the host cell genome at random or specific sites (for review see Samulski, R. J., *Current Opinion in Genetics and Development* 3:74-80 (1993); Muzyczka, N., *Curr. Top. Microbiol. Immunol.* 158:97-129 (1992)). HSV, adenovirus and rAAV vectors are all packaged in stable particles. Retrovirus vectors can accommodate 7-8 kb of foreign DNA and integrate into the host cell genome, but only in mitotic cells, and particles are relatively unstable with low titers. Recent studies have demonstrated that elements from different viruses can be combined to increase the delivery capacity of vectors. For example, incorporation of elements of the HIV virion, including the matrix protein and integrase, into retrovirus vectors allows transgene cassettes to enter the nucleus of non-mitotic, as well as mitotic cells and potentially to integrate into the genome of these cells (Naldini, L. *et al.*, *Science* 272:263-267 (1996)); and inclusion of the vesicular stomatitis virus envelope glycoprotein (VSV-G) increases stability of retrovirus particles (Emi, N. *et al.*, *J. Virol.* 65:1202-1207 (1991)).

- [00106] HSV-1 is a double-stranded DNA virus which is replicated and transcribed in the nucleus of the cell. HSV-1 has both a lytic and a latent cycle. HSV-1 has a wide host range, and infects many cell types in mammals and birds (including chicken, rat, mice monkey, and human) Spear *et al.*, *DNA Tumor Viruses*, J. Tooze, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1981, pp. 615-746). HSV-1 can lytically infect a wide variety of cells including neurons, fibroblasts and macrophages. In addition, HSV-1 infects post-mitotic neurons in adult animals and can be maintained indefinitely in a latent state. Stevens, *Current Topics in Microbiology and Immunology* 70:31(1975). Latent HSV-1 is capable of expressing genes.
- [00107] AAV also has a broad host range and most human cells are thought to be injectable. The host range for integration is believed to be equally broad. AAV is a single stranded DNA parvovirus endogenous to the human population, making it a suitable gene therapy vector candidate. AAV is not associated with any disease, therefore making it safe for gene transfer applications (Cukor *et al.*, *The Parvoviruses*, Ed. K. I. Berns, Plenum, N.Y., (1984) pp. 33-36; Ostrove *et al.*, *Virology* 113: 521 (1981)). AAV integrates into the host genome upon infection so that transgenes can be expressed indefinitely (Kotin *et al.*, *Proc. Natl. Acad. Sci. USA* 87: 221 (1990); Samulski *et al.*, *Embo J.* 10: 3941 (1991)). Integration of AAV into the cellular genome is independent of cell replication which is particularly important since AAV can thus transfer genes into quiescent cells (Lebkowski *et al.*, *Mol. Cell. Biol.* 8:3988 (1988)).
- [00108] Both HSV and AAV can deliver genes to dividing and non-dividing cells. In general, HSV virions are considered more highly infectious than AAV virions, with a ratio of virus particles: infectious units in the range of 10 for HSV (Browne, H. *et al.*, *J. Virol.* 70:4311-4316 (1996)) and up to thousands for AAV (Snyder, R. O. *et al.*, *In Current Protocols in Human Genetics*, Eds. Dracopoli, N. *et al.*, John Wiley and Sons: New York (1996), pp. 1-24), and both having a broad species range. Still, each virion has specific tropisms which will affect the efficiency of infection of specific cell types. The recent

identification of a membrane receptor for HSV-1 which is a member of the tumor necrosis factor alpha family (Montgomery, R. I. *et al.*, 21st Herpes Virus Workshop Abstract #167 (1996)) indicates that the distribution of this receptor will affect the relative infectability of cells, albeit most mammalian cell types appear to be injectable with HSV-1. AAV also has a very wide host and cell type range. The cellular receptor for AAV is not known, but a 150 kDA glycoprotein has been described whose presence in cultured cells correlates with their ability to bind AAV (Mizukami, H. *et al.*, *Virology* 217:124-130 (1996)).

[00109] Techniques for the formation of such vectors are well-known in the art, and are generally described in "Working Toward Human Gene Therapy," Chapter 28 in *Recombinant DNA, 2nd Ed.*, Watson, J. D. *et al.*, eds., New York: Scientific American Books, pp. 567-581 (1992). In addition, general methods for construction of gene therapy vectors and the introduction thereof into affected animals for therapeutic purposes may be found in the above-referenced publications, the disclosures of which are specifically incorporated herein by reference in their entirety.

[00110] In one general method, vectors comprising polynucleotides encoding PTH derivative gene are directly introduced into the cells or tissues of the affected individual, preferably by injection, inhalation, ingestion or introduction into a mucous membrane via solution; such an approach is generally referred to as "in vivo" gene therapy. Alternatively, cells or tissues, e.g., hematopoietic cells from bone marrow, may be removed from the affected animal and placed into culture according to methods that are well-known to one of ordinary skill in the art; the vectors comprising the polynucleotides may then be introduced into these cells or tissues by any of the methods described generally above for introducing isolated polynucleotides into a cell or tissue, and, after a sufficient amount of time to allow incorporation of the polynucleotides, the cells or tissues may then be re-inserted into the affected animal or a second animal in need of treatment. Since the introduction of the DNA of interest is performed outside of the body

of the affected animal, this approach is generally referred to as "ex vivo" gene therapy.

[00111] For both in vivo and ex vivo gene therapy, the polynucleotides of the invention may alternatively be operatively linked to a regulatory DNA sequence, which may be a heterologous regulatory DNA sequence, to form a genetic construct as described above. This genetic construct may then be inserted into a vector, which is then directly introduced into the affected animal in an in vivo gene therapy approach, or into the cells or tissues of the affected animal in an ex vivo approach. In another preferred embodiment, the genetic construct may be introduced into the cells or tissues of the animal, either in vivo or ex vivo, in a molecular conjugate with a virus (e.g., an adenovirus) or viral components (e.g., viral capsid proteins).

[00112] The above approaches result in (a) homologous recombination between the nucleic acid molecule and the defective gene in the cells of the affected animal; (b) random insertion of the gene into the host cell genome; or (c) incorporation of the gene into the nucleus of the cells where it may exist as an extrachromosomal genetic element. General descriptions of such methods and approaches to gene therapy may be found, for example, in U.S. Pat. No. 5,578,461; WO 94/12650; and WO 93/09222.

[00113] Alternatively, transfected host cells, which may be homologous or heterologous, may be encapsulated within a semi-permeable barrier device and implanted into the affected animal, allowing passage of for example the PTH polypeptide derivative into the tissues and circulation of the animal but preventing contact between the animal's immune system and the transfected cells (see WO 93/09222).

#### *Utility and Administration of Compounds of the Invention*

[00114] Compounds of the invention or derivatives thereof have multiple uses. These include, *inter alia*, agonists or antagonists of the PTH receptor, prevention and treatment of a variety of mammalian conditions manifested by loss of bone mass, diagnostic probes, antigens to prepare antibodies for use as



diagnostic probes and even as molecular weight markers. Being able to specifically substitute one or more amino acids in the PTH polypeptide permits construction of specific molecular weight polypeptides as required.

[00115] In particular, the compounds of this invention are indicated for the prophylaxis and therapeutic treatment of osteoporosis and osteopenia in humans. Furthermore, the compounds of this invention are indicated for the prophylaxis and therapeutic treatment of other bone diseases. The compounds of this invention are also indicated for the prophylaxis and therapeutic treatment of hypoparathyroidism. Finally, the compounds of this invention are indicated for use as agonists for fracture repair and as antagonists for hypercalcemia.

[00116] In general, compounds of for example, SEQ ID NO: 1 or derivatives thereof, or salts thereof, are administered in amounts between about 0.01 and 1  $\mu\text{g/kg}$  body weight per day, preferably from about 0.07 to about 0.2  $\mu\text{g/kg}$  body weight per day. For a 50 kg human female subject, the daily dose of biologically active compound is from about 0.5 to about 50  $\mu\text{g}$ s, preferably from about 3.5 to about 10  $\mu\text{g}$ s. In other mammals, such as horses, dogs, and cattle, higher doses may be required. This dosage may be delivered in a conventional pharmaceutical composition by a single administration, by multiple applications, or via controlled release, as needed to achieve the most effective results, preferably one or more times daily by injection. For example, this dosage may be delivered in a conventional pharmaceutical composition by nasal insufflation.

[00117] The selection of the exact dose and composition and the most appropriate delivery regimen will be influenced by, *inter alia*, the pharmacological properties of the selected compounds of the invention, the nature and severity of the condition being treated, and the physical condition and mental acuity of the recipient.

[00118] Representative preferred delivery regimens include, without limitation, oral, parenteral, subcutaneous, transcutaneous, intramuscular and intravenous, rectal, buccal (including sublingual), transdermal, and intranasal insufflation.

**[00119]**     Pharmaceutically acceptable salts retain the desired biological activity of the compounds of the invention without toxic side effects. Examples of such salts are (a) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; and salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalene disulfonic acids, polygalacturonic acid and the like; (b) base addition salts formed with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, and the like; or with an organic cation formed from N,N'-dibenzylethylenediamine or ethylenediamine; or (c) combinations of (a) and (b), e.g., a zinc tannate salt and the like. Pharmaceutically acceptable buffers include but are not limited to saline or phosphate buffered saline. Also included in these solutions may be acceptable preservative known to those of skill in the art.

**[00120]**     A further aspect of the present invention relates to pharmaceutical compositions comprising as an active ingredient compounds of the invention or derivatives thereof of the present invention, or pharmaceutically acceptable salt thereof, in admixture with a pharmaceutically acceptable, non-toxic carrier. As mentioned above, such compositions may be prepared for parenteral (subcutaneous, transcutaneous, intramuscular or intravenous) administration, particularly in the form of liquid solutions or suspensions; for oral or buccal administration, particularly in the form of tablets or capsules; for rectal, transdermal administration; and for intranasal administration, particularly in the form of powders, nasal drops or aerosols.

**[00121]**     The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., (1985), incorporated herein by reference. Formulations for parenteral administration

may contain as excipients sterile water or saline, alkylene glycols such as propylene glycol, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. For oral administration, the formulation can be enhanced by the addition of bile salts or acylcarnitines. Formulations for nasal administration may be solid and may contain excipients, for example, lactose or dextran, or may be aqueous or oily solutions for use in the form of nasal drops or metered spray. For buccal administration typical excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like.

**[00122]** When formulated for the most preferred route of administration, nasal administration, the absorption across the nasal mucous membrane may be enhanced by surfactant acids, such as for example, glycocholic acid, cholic acid, taurocholic acid, ethocholic acid, deoxycholic acid, chenodeoxycholic acid, dehydrocholic acid, glycodeoxycholic acid, cyclodextrins and the like in an amount in the range between about 0.2 and 15 weight percent, preferably between about 0.5 and 4 weight percent, most preferably about 2 weight percent.

**[00123]** Delivery of the compounds of the present invention to the subject over prolonged periods of time, for example, for periods of one week to one year, may be accomplished by a single administration of a controlled release system containing sufficient active ingredient for the desired release period. Various controlled release systems, such as monolithic or reservoir-type microcapsules, depot implants, osmotic pumps, vesicles, micelles, liposomes, transdermal patches, iontophoretic devices and alternative injectable dosage forms may be utilized for this purpose. Localization at the site to which delivery of the active ingredient is desired is an additional feature of some controlled release devices, which may prove beneficial in the treatment of certain disorders.

**[00124]** One form of controlled release formulation contains the polypeptide or its salt dispersed or encapsulated in a slowly degrading, non-toxic, non-antigenic polymer such as copoly(lactic/glycolic) acid, as described in the

pioneering work of Kent, Lewis, Sanders, and Tice, U.S. Pat. No. 4,675,189, incorporated by reference herein. The compounds or, preferably, their relatively insoluble salts, may also be formulated in cholesterol or other lipid matrix pellets, or silastomer matrix implants. Additional slow release, depot implant or injectable formulations will be apparent to the skilled artisan. See, for example, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson ed., Marcel Dekker, Inc., New York, 1978, and R. W. Baker, Controlled Release of Biologically Active Agents, John Wiley & Sons, New York, 1987, incorporated by reference herein.

[00125] Like PTH, the PTH variants may be administered in combination with other agents useful in treating a given clinical condition. When treating osteoporosis and other bone-related disorders for example, the PTH variants may be administered in conjunction with a dietary calcium supplement or with a vitamin D analog (see U.S. Pat. No. 4,698,328). Alternatively, the PTH variant may be administered, preferably using a cyclic therapeutic regimen, in combination with bisphosphonates, as described for example in U.S. Pat. No. 4,761,406, or in combination with one or more bone therapeutic agents such as, without limitation, calcitonin and estrogen.

*Receptor-Signaling Activities of Compounds of the Invention or Derivatives Thereof*

[00126] A crucial step in the expression of hormonal action is the interaction of hormones with receptors on the plasma membrane surface of target cells. The formation of hormone-receptor complexes allows the transduction of extracellular signals into the cell to elicit a variety of biological responses.

[00127] Polypeptides of the invention may be screened for their agonistic or antagonistic properties using the cAMP accumulation assay. Cells expressing PTH-1 receptor on the cell surface are incubated with native PTH(1-84) for 5 to 60 minutes at 37°C., in the presence of 2 mM IBMX (3-isobutyl-1-methylxanthine, Sigma, St. Louis, Mo.). Cyclic AMP accumulation is measured by specific radio-immunoassay. A compound of the invention that competes with

native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, and that inhibits the effect of native PTH(1-84) or PTH(1-34) on cAMP accumulation, is considered a competitive antagonist. Such a compound would be useful for treating hypercalcemia.

**[00128]** Conversely, a compound of the invention or a derivative thereof that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, but which still prevents native PTH(1-84) or PTH(1-34) activation of cAMP accumulation (presumably by blocking the receptor activation site) is considered a non-competitive antagonist. Such a compound would be useful for treating hypercalcemia.

**[00129]** A compound of the invention or a derivative thereof that competes with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, and which stimulates cAMP accumulation in the presence or absence of native PTH(1-84) or PTH(1-34) is a competitive agonist. A compound of the invention or a derivative thereof that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor but which is still capable of stimulating cAMP accumulation in the presence or absence of native PTH(1-84) or PTH(1-34), or which stimulates a higher cAMP accumulation than that observed by a compound of the invention or a derivative thereof alone, would be considered a non-competitive agonist.

**[00130]** Likewise, polypeptides of the invention may be screened for their agonistic or antagonistic properties using the inositol phosphate accumulation assay. Cells expressing PTH-1 receptor on the cell surface are incubated with native PTH(1-84), and inositol phosphate accumulation is measured by specific radio-immunoassay. A compound of the invention that competes with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, and that inhibits the effect of native PTH(1-84) or PTH(1-34) on inositol phosphate accumulation, is considered a competitive antagonist. Such a compound would be useful for treating hypercalcemia.

**[00131]** Conversely, a compound of the invention or a derivative thereof that does not compete with native PTH(1-84) or PTH(1-34) for binding to the

PTH-1 receptor, but which still prevents native PTH(1-84) or PTH(1-34) activation of inositol phosphate accumulation (presumably by blocking the receptor activation site) is considered a non-competitive antagonist. Such a compound would be useful for treating hypercalcemia.

[00132] A compound of the invention or a derivative thereof that competes with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, and which stimulates inositol phosphate accumulation in the presence or absence of native PTH(1-84) or PTH(1-34) is a competitive agonist. A compound of the invention or a derivative thereof that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor but which is still capable of stimulating inositol phosphate accumulation in the presence or absence of native PTH(1-84) or PTH(1-34), or which stimulates a higher inositol phosphate accumulation than that observed by a compound of the invention or a derivative thereof alone, would be considered a non-competitive agonist.

*Therapeutic Uses of Compounds of the Invention or Derivatives Thereof*

[00133] Some forms of hypercalcemia and hypocalcemia are related to the interaction between PTH and PTHrP and the PTH-1 and PTH-2 receptors. Hypercalcemia is a condition in which there is an abnormal elevation in serum calcium level; it is often associated with other diseases, including hyperparathyroidism, osteoporosis, carcinomas of the breast, lung and prostate, epidermoid cancers of the head and neck and of the esophagus, multiple myeloma, and hypernephroma. Hypocalcemia, a condition in which the serum calcium level is abnormally low, may result from a deficiency of effective PTH, e.g., following thyroid surgery.

[00134] Nucleic acids of the invention which encode compounds of the invention or derivatives thereof may also be linked to a selected tissue-specific promoter and/or enhancer and the resultant hybrid gene introduced, by standard methods (e.g., as described by Leder *et al.*, U.S. Pat. No. 4,736,866, herein incorporated by reference), into an animal embryo at an early developmental stage (e.g., the fertilized oocyte stage), to produce a transgenic

animal which expresses elevated levels of compounds of the invention or derivatives thereof in selected tissues (e.g., the osteocalcin promoter for bone). Such promoters are used to direct tissue-specific expression of compounds of the invention or derivatives thereof in the transgenic animal.

**[00135]** In addition, any other amino-acid substitutions of a nature, which do not destroy the ability of the PTH derivative to antagonize or agonize the PTH-1/PTH-2 receptor (as determined by assays known to the skilled artisan and discussed below) are included in the scope of the present invention.

**[00136]** By "agonist" is intended a ligand capable of enhancing or potentiating a cellular response mediated by the PTH-1 receptor. By "antagonist" is intended a ligand capable of inhibiting a cellular response mediated by the PTH-1 receptor. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit such a cellular response can be determined using art-known protein ligand/receptor cellular response or binding assays, including those described elsewhere in this application.

**[00137]** In accordance with yet a further aspect of the invention, there is provided a method for treating a medical disorder that results from altered or excessive action of the PTH-1 receptor, comprising administering to a patient therapeutically effective amount of a compound of the invention or a derivative thereof sufficient to inhibit activation of the PTH-1 receptor of said patient.

**[00138]** In this embodiment, a patient who is suspected of having a disorder resulting from altered action of the PTH-1 receptor may be treated using compounds of the invention or derivatives thereof of the invention which are a selective antagonists of the PTH-1 receptor. Such antagonists include compounds of the invention or derivatives thereof of the invention which have been determined (by the assays described herein) to interfere with PTH-1 receptor-mediated cell activation or other derivatives having similar properties.

**[00139]** To administer the antagonist, the appropriate compound of the invention or a derivative thereof is used in the manufacture of a medicament,

generally by being formulated in an appropriate carrier or excipient such as, e.g., physiological saline, and preferably administered intravenously, intramuscularly, subcutaneously, orally, or intranasally, at a dosage that provides adequate inhibition of a compound of the invention or a derivative thereof binding to the PTH-1 receptor. Typical dosage would be 1 ng to 10 mg of the peptide per kg body weight per day.

[00140] In accordance with yet a further aspect of the invention, there is provided a method for treating osteoporosis, comprising administering to a patient a therapeutically effective amount of a compound of the invention or a derivative thereof, sufficient to activate the PTH-1 receptor of said patient. Similar dosages and administration as described above for the PTH/PTHrP antagonist, may be used for administration of a PTH/PTHrP agonist, e.g., for treatment of conditions such as osteoporosis, other metabolic bone disorders, and hypoparathyroidism and related disorders.

[00141] It will be appreciated to those skilled in the art that the invention can be performed within a wide range of equivalent parameters of composition, concentration, modes of administration, and conditions without departing from the spirit or scope of the invention or any embodiment thereof.

#### *Interactions of Parathyroid Hormone and Parathyroid Hormone Receptors*

[00142] Understanding the mechanisms by which peptide hormones and G protein-coupled receptors interact is of fundamental biological importance. Parathyroid hormone (PTH), a key regulator of calcium, phosphate and bone metabolism, interacts with a class-2 GPCR, the PTH receptor or PTHR, which is expressed in bone and kidney cells. Each of the 15 or so class-2 GPCRs binds a peptide hormone with a pharmacophoric region that spans about 30 amino acids in peptide chain length. These peptides are generally thought to interact with their receptor via a multi-step mechanism that involves ligand contacts to both the receptor's amino-terminal extracellular (N) domain, and to its juxtamembrane (J) region that contains the extracellular loops and seven transmembrane domain helices. For the PTHR, which also binds the



developmental protein, PTH-related protein, understanding the molecular mechanisms that underlie the ligand-interaction process has direct implications for human health, as PTH agonist ligands, such as PTH(1-34) and PTH(1-31) can potently stimulate bone formation and are thus effective therapies for treating osteoporosis (Neer, R., Arnaud, *et al.* (2001) *N.E.JM* 344, 1434-1441; Whitfield, J. F., *et al.* (1997) *Calcif Tissue Int* 60, 26-29).

[00143] An extensive body of data derived largely from mutational and photoaffinity cross-linking studies suggest that the mechanism by which PTH(1-34), or as used in this study, PTH(1-31), interacts with the PTHR involves two principal, and to some extent, autonomous, components (reviewed in Gensure, R. C., *et al.* (2005) *Biochem Biophys Res Commun* 328, 666-78). The first component is an interaction between the ligand's principal receptor-binding domain, contained within the carboxyl-terminal portion of the molecule (Rosenblatt, M., *et al.* (1980) *Endocrinology* 107, 545-550), and the receptor's amino-terminal extracellular domain, a presumed disulfide-stabilized globular structure (Grace, C. R., *et al.* (2004) *Proc Natl Acad Sci USA* 101:12836-41). The second is an interaction between the principal signaling domain of the ligand, contained within the amino-terminal portion of the molecule, and the receptor's juxtamembrane region of the heptahelical core. By this model, the initial docking of the ligand to the PTHR N domain anchors the ligand to the receptor and thus enables the amino-terminal portion of the ligand to engage the receptor's J domain and induce the conformational changes involved in receptor activation (Hoare, S., *J. Biol. Chem* 276, 7741-7753).

[00144] Solution phase NMR studies generally show that the carboxyl-terminal binding domain portion of a PTH ligand forms a stable  $\alpha$ -helix, whereas the amino-terminal portion of the ligand is largely disordered (Chen, Z., *et al.* (2000) *Biochemistry* 39, 12766-12777; Pellegrini, M., *et al.* (1998) *J. Biol. Chem.* 273, 10420-10427). Functional studies with modified amino-terminal PTH fragment analogs suggest that binding of the N-terminal domain of the ligand to the receptor's J domain is coupled to a folding of that ligand domain

into an  $\alpha$ -helix. Thus, the cAMP-stimulating potency of the native PTH(1-14) fragment is greatly improved by substitutions, such as Aib at positions 1 and 3, that rigidify the peptide into an  $\alpha$ -helix (Shimizu, M., *et al.* (2001) *Endocrinology* 142, 3068-3074; Tsomaia, N., *et al.* (2004) *Biochemistry* 43:690-9). Moreover, amino-terminal fragments such as [Aib<sup>1,3</sup>,Gln<sup>10</sup>,Har<sup>11</sup>,Ala<sup>12</sup>,Trp<sup>14</sup>]PTH(1-14)NH<sub>2</sub> stimulate full and potent cAMP responses in cells expressing a mutant PTHR construct, PTHR-delNt, that lacks the receptor's N- domain, and on which unmodified PTH(1-34) is only weakly active (Shimizu, N., *et al.* (2001) *J Biol Chem* 276, 49003-49012; Shimizu, M., *et al.* (2002) *Biochemistry* 41:13224-13233.), due to the absence of N domain interactions. These findings with modified PTH(1-14) analogs and PTHR-delNt also serve to illustrate the autonomous potential of the J domain component of the PTH-PTHr interaction mechanism.

[00145] For the native hormone acting on the intact PTHR, the ligand's carboxyl-terminal binding domain, contained within the (Ser<sup>17</sup>-Val<sup>31</sup>) region, clearly plays a key role in the interaction process. Within this domain, functional studies have identified Arg<sup>20</sup>, Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup> as important PTHR-binding determinants (Gardella, T. J., *et al.* (1993) *Endocrinology* 132:2024-2030; Oldenburg, K. R., *et al.* (1996) *Journal Of Biological Chemistry* 271:17582-17591; Barbier, J., *et al.* (2001) *Biochemistry* 40:8955-8961; Reidhaar-Olson, J., *et al.* (2000) *Mol Cell Endocrinol* 160:135-147). The  $\alpha$ -helix contained within this (17-31) segment has amphiphilic character (Neugebauer, W., (1992) *Biochemistry* 31:2056-2063; Epand, R. E. (1983) *Mol and Cell Biol* 57, 41-47; Barbier, J. R., *et al.* (2005) *J Biol Chem.* 280:23771-23777), the hydrophobic face of which, formed largely by Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup>, appears to contribute most importantly to the PTHR-binding process, according to the above substitution studies. Although the two-domain model of the PTH-PTHr interaction mechanism predicts that these residues interact specifically with the PTHR N domain, this has not been firmly established. Moreover, the recent finding that [Lys<sup>27</sup>(Bp)<sub>2</sub>]PTH(1-34)NH<sub>2</sub>, modified with photo-reactive benzophenone groups on the side chain amines

of Lys<sup>27</sup>, cross-links to the first extracellular loop of the PTHR (Greenberg, Z., (2000) *Biochemistry* 39:8142-8152) raises the possibility that some interaction between the carboxyl-terminal helix and the PTHR J domain can occur. Consistent with this possibility, recent functional studies have found that methylation of certain backbone nitrogen atoms in the (17-31) domain of PTH(1-31) impairs, albeit modestly, the cAMP-signaling activity of the ligand in cells expressing PTHR-delNt (Barbier, J. R., *et al.* (2005) *J Biol Chem.* 280:23771-23777). Thus, the mechanisms by which the carboxyl-terminal binding domain of PTH contributes to the PTHR interaction process are presently not well understood.

[00146] For the PTHR, the new PTH analogs presented here can be used to probe receptor mutants altered at candidate sites in the N domain, to identify, via epistatic, rescue effects, sites of functionally relevant intermolecular interaction.

[00147] Having now fully described the invention, the same will be more readily understood by reference to specific examples which are provided by way of illustration, and are not intended to be limiting of the invention, unless herein specified.

[00148] It should be understood that these conditions are not meant to be definitive or limiting and may be adjusted as required by those of ordinary skill in the art to accomplish the desired objective.

## EXAMPLES

### *Introduction to Examples*

[00149] Studies were designed to further examine the mechanisms of interaction between the parathyroid hormones and hormone receptors. The roles that the amino acid side chains in this domain play in the receptor-binding process, the functional involvement of the putative amphiphilic  $\alpha$ -helix, and the potential for interactions to the PTHR J domain were studied.

The strategy was to introduce a number of substitutions, conservative and non-conservative, in the (17-31) region of PTH(1-31) analogs and assess their effects on binding to both the intact PTHR and to PTHR-delNt. The analysis of binding to the latter receptor was made possible by using  $^{125}\text{I}$ -[Aib<sup>1,3</sup>,Nle<sup>8</sup>,Gln<sup>10</sup>,Har<sup>11</sup>,Trp<sup>14</sup>,Tyr<sup>15</sup>]PTH(1-15) as a tracer radioligand and expressing the truncated mutant PTHR in cells co-transfected with a negative-dominant Gas subunit that couples more efficiently to the receptor, and thus improves the overall binding signal (Dean, T., (2006) *Mol Endocrinol* 20:931-942). This general approach enabled, for the first time, the study of effects of modifications in the carboxyl-terminal binding domain of relatively unmodified PTH ligands on interaction with the PTHR J domain, as defined by the PTHR-delNt construct. The overall results help define the roles that specific amino acid side-chains in the carboxyl-terminal domain of PTH play in the PTHR-binding process, and shed new light on the overall topology of the PTH-PTHr complex.

#### *Materials and Methods*

[00150] *Peptide synthesis* —Peptides were based on the human PTH(1-31)NH<sub>2</sub> sequence (SVSEIQLMHNLGKHLNSMERVEWLRKKLQDV-NH<sub>2</sub> (SEQ ID NO:13)). Alanine substitutions were incorporated into this PTH(1-31)NH<sub>2</sub> scaffold. To augment binding to PTHR-delNt, the Ser<sup>1</sup>→Ala (Shimizu, M., *et al.* (2000) *J. Biol. Chem.* 275:21836-21843) and Glu<sup>19</sup>→Arg (Shimizu, M., *et al.* (2002) *Biochemistry* 41:13224-13233) substitutions were introduced to yield [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub>. This scaffold peptide was used for studies involving the Cha and Glu substitutions. These PTH(1-31)NH<sub>2</sub>, and [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> peptides, and their Ala-, Glu- and Cha-substituted derivatives were synthesized by the M.G.H. Biopolymer core facility using a multiple peptide synthesizer (Advanced Chemtech Model 396 MBS) and solid-phase, Fmoc-based, coupling/deprotection chemistry. Peptides were desalted using a C18 Sep-Pak cartridge and acetonitrile-based elution. The Ala-substituted PTH(1-31) analogs, shown by HPLC analysis to be at least

90% homogeneous at this stage, were used without further purification. The Cha- and Glu-substituted peptides were further purified by reversed-phase HPLC. Analogs of PTH(1-31)NH<sub>2</sub> with additional substitutions at position 20 were prepared as part of the previous studies by Barbier *et al.* ((2001) *Biochemistry* 40:8955-8961). Lyophilized peptides were reconstituted as stock solutions in 10 mM acetic acid. All peptides were verified by analytical HPLC, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and amino acid analysis. Peptide concentrations were established by amino acid analysis. The radioligands <sup>125</sup>I-[Nle<sup>8,21</sup>,Tyr<sup>34</sup>]ratPTH(1-34)NH<sub>2</sub> and <sup>125</sup>I-[Aib<sup>1,3</sup>,Nle<sup>8</sup>,Gln<sup>10</sup>,Har<sup>11</sup>,Ala<sup>12</sup>,Trp<sup>14</sup>,Tyr<sup>15</sup>]ratPTH(1-15)NH<sub>2</sub>(<sup>125</sup>I-PTH(1-34)NH<sub>2</sub> and <sup>125</sup>I-[Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub>, respectively) were prepared by the oxidative chloramine-T procedure using Na<sup>125</sup>I (specific activity: 2,200 Ci/mmol, Perkin Elmer/NEN Life Science Products, Boston, MA) and were purified by reversed-phase HPLC.

[00151] *Circular Dichroism* - CD spectra were obtained on a JASCO J-600 spectropolarimeter at 20°C. Four spectra were averaged and the data smoothed by the JASCO software. The instrument was calibrated with ammonium (+)-10-camphorsulfonate. Peptide concentrations were verified from the absorption at 280 nm, using an extinction coefficient of 5700 M<sup>-1</sup> for the single tryptophan. Data are expressed as the number of helical residues per peptide chain, as calculated from  $-[\theta]_{222} \times 30/28,000$ , where  $[\theta]_{222}$  is the mean residue ellipticity ( $[\theta]$ ) at 222 nm, as was described previously (Barbier, J. R., *et al.* (2005) *J Biol Chem.* 280:23771-23777).

[00152] *Cell Culture* - Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (HyClone, Logan UT), 100 units/ml penicillin G, and 100 ug/ml streptomycin penicillin G/streptomycin (Invitrogen Corp. Carlsbad, CA). For binding and cAMP studies performed with the intact PTHR, the HKRK-B7 and ROS 17/2.8 cell lines were used. HKRK-B7 cells are derived from the porcine kidney cell line LLC-PK1 and

express, via stable DNA transfection, the wild-type human PTHR at an approximate surface density of 950,000 PTH-binding sites per cell. ROS 17/2.8 cells are rat osteosarcoma cells and express the endogenous PTHR at an approximate surface density of 80,000 PTH-binding sites per cell. The cells were plated and assayed in 24-well plates.

**[00153]** PTHR-delNt is a derivative of the human PTHR that is deleted for most (A1a<sup>24</sup>-Arg<sup>181</sup>) of the amino-terminal extracellular domain (Shimizu, M., *et al.* (2001) *Endocrinology* 142:3068-3074). This PTHR construct was expressed in COS-7 cells via transient DNA transfection. For cAMP assays, the cells were transfected in 24-well plates using 0.2 µg of plasmid DNA and 0.6 µl of Fugene-6 reagent (Roche Diagnostics, Indianapolis IN) per well, and assays were performed on the intact cells three to four days after transfection. For binding assays, cell membranes were prepared from transfected COS-7 cells. To increase the maximal binding of <sup>125</sup>I-[Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub> to PTHR-delNt in these membranes, the cells were cotransfected with a negative dominant mutant Gα<sub>s</sub> protein (Gα<sub>s</sub><sup>ND</sup>). This mutant Gα<sub>s</sub> subunit is thought to couple to cognate receptors, and thus stabilize high affinity receptor conformations, more efficiently than does wild-type Gα<sub>s</sub>, without increasing basal cAMP levels (Berlot, C. H. (2002) *J Biol Chem* 277:21080-5). A precursor of this mutant Gα<sub>s</sub>, Gα<sub>s</sub>(α3β5), was recently used to increase binding of <sup>125</sup>I-[Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub> to PTHR-delNt in COS-7 cell membranes (Dean, T., (2006) *Mol Endocrinol* 20:931-942). Gα<sub>s</sub><sup>ND</sup>, which contains the same five amino acid replacement of corresponding Gα<sub>i</sub> residues in the α3β5 loop as Gα<sub>s</sub>(α3β5), plus the point mutations of Gly<sup>226</sup> to Ala, which increases affinity for Gβ/γ, and Ala<sup>366</sup> to Ser, which decreases affinity for GDP, yields approximately two-fold higher levels of specific binding of <sup>125</sup>I-[Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub> than does Gα<sub>s</sub>(α3β5) (data not shown). For the current membrane preparations, COS-7 cells were co-transfected in six-well plates using 1 µg of each plasmid DNA encoding PTHR-delNt and Gα<sub>s</sub><sup>ND</sup>, and 6µl of Fugene-6 reagent (Roche Diagnostics, Indianapolis IN) per well. Cells

were harvested three days after transfection, and membranes were prepared as described (Dean, T., *et al.* (2006) *Mol Endocrinol* 20:931-942).

[00154] *Receptor binding* - Binding to the wild-type human PTHR in intact HKRK-B7 cells and ROS 17/2.8 cells was assessed using  $^{125}\text{I}$ -PTH(1-34) as a tracer radioligand, as described (Shimizu, M., (2001) *Endocrinology* 142:3068-3074). In brief, the cells in 24-well plates (about 500,000 cells per well) were incubated in binding buffer containing radioligand (about 100,000 cpm per well) with or without unlabeled peptide ligand ( $3 \times 10^{-9}$  to  $1 \times 10^{-5}$  M) for four hours at  $15^\circ\text{C}$ . The binding mixture was then removed by aspiration, the cells were rinsed three times with binding buffer, lysed in 1 M NaOH, and the entire lysate was counted for gamma-irradiation in a gamma counter. Binding to PTHR-delNt in COS-7 cell membranes was assessed in 96-well, vacuum filtration plates (Multiscreen-Durapore HV, 0.65  $\mu\text{M}$  membranes, Millipore Corp., Millford, MA) using  $^{125}\text{I}$ -[Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub> as a tracer radioligand, as described (Dean, T., (2006) *Mol Endocrinol* 20:931-942). In brief, cell membranes (20  $\mu\text{g}$ /well) were incubated in membrane binding buffer containing radioligand (30,000 cpm/well) with or without unlabeled peptide ligand ( $3 \times 10^{-9}$  to  $1 \times 10^{-5}$  M) for 90 minutes at  $21^\circ\text{C}$ . The plates were then subject to rapid vacuum filtration, the filters were washed once with buffer, detached from the plate and counted for gamma-irradiation in a gamma counter. Non-specific binding was defined as the binding observed in the presence of  $1 \times 10^{-6}$  M PTH(1-31)NH<sub>2</sub> for HKRK-B7 and ROS 17/2.8 cells, and  $1 \times 10^{-6}$  M [Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub> for PTHR-delNt. Specifically bound radioactivity was calculated as a percentage of the radioactivity specifically bound in the absence of competing ligand.

[00155] *Stimulation of intracellular cAMP and inositol phosphate* - The capacities of the ligands to stimulate adenylyl cyclase activity were assessed in intact ROS 17/2.8 cells, as described (Shimizu, M., (2001) *Endocrinology* 142:3068-3074). In brief, cells in 24-well plates were incubated in binding buffer containing the phosphodiesterase inhibitor, IBMX, with or without a peptide ligand ( $3 \times 10^{-9}$  to  $1 \times 10^{-5}$  M) for 30 minutes at room temperature. The

medium was removed and the cells were lysed by adding 50 mM HCl and freezing the plate on dry ice. The cAMP in the thawed lysate was then quantified by radioimmuno assay. The stimulation of production of inositol phosphates ( $IP_1+IP_2+IP_3$ ) was assessed in COS-7 cells transfected with the intact human PTHR, as described previously (Shimizu, M., (2001) *Endocrinology* 142:3068-3074). In brief, intact transfected COS-7 cells in 24-well plates were labeled with  $^3H$ -myo-inositol (specific activity: 25 Ci/mmol, Perkin Elmer/NEN Life Science Products, Boston, MA) for 16 hours, treated for 30 minutes with ligand in the presence of  $LiCl_2$  (30 mM), treated with ice cold trichloroacetic acid (5%) for two hours; the acid lysates were then ether extracted, separated by ion-exchange chromatography, and eluted  $^3H$ -inositol phosphates were quantified by liquid scintillation counting.

[00156] *Data and statistical calculations* - Data were processed for curve fitting and derivation of  $IC_{50}$  and  $EC_{50}$  values using least-squares, nonlinear regression analysis and the four parameter logistic equation:  $y = y_{min} + (V_{max} - y_{min}) / (1 + (IC_{50}/x)^n)$ , where  $y$ ,  $y_{min}$  and  $y_{max}$  are the observed, minimum and maximum response values, respectively;  $x$  is ligand concentration, and  $n$  is the slope factor. In cases where incomplete inhibition of binding occurred: i.e. with certain PTH(1-31) analogs binding to PTHR-delNt, the curve fits and  $IC_{50}$  values were derived by extrapolating the data to the maximum inhibition attained by  $[Aib^{1,3}, M]PTH(1-15)NH_2$ . Paired data sets were statistically compared using a two-tailed Student's t-test, assuming unequal variances for the two sets.

#### EXAMPLE 1

[00157] *Alanine-Scan of the PTH(17-31) domain* - Each residue in the (17-31) region of PTH(1-31) $NH_2$  was individually replaced with alanine and the effects of the substitutions on binding to the intact human PTHR stably expressed in HKRK-B7 cells was assessed. Competition binding studies were performed using whole cells and  $^{125}I$ -PTH(1-34) ( $^{125}I$ -[Nle $^{8,21}$ , Tyr $^{34}$ ]ratPTH(1-



34)NH<sub>2</sub>) as a tracer radioligand. The unsubstituted parental PTH(1-31)NH<sub>2</sub> peptide fully inhibited the binding of this tracer with an IC<sub>50</sub> of 68±10 nM (Fig. 1A; Table 1). The various alanine substitutions had a range of effects on this apparent PTHR-binding affinity. Most dramatically, the Arg<sup>20</sup> to Ala substitution, abolished detectable binding altogether (FIG. 1A). The alanine substitutions at Trp<sup>23</sup> and Leu<sup>24</sup> reduced binding affinity by 19- and 12-fold, respectively ( $P \leq 0.05$ ), and those at Val<sup>21</sup>, Arg<sup>25</sup>, Lys<sup>27</sup>, Leu<sup>28</sup> and Val<sup>31</sup> reduced affinity by three- to four-fold (FIGS. 1A and B; Table 1). The remaining alanine substitutions altered binding affinity by two-fold or less. The alanine substitutions at Glu<sup>19</sup>, Glu<sup>22</sup> and Gln<sup>29</sup> each produced a small ( $\leq$  two-fold, enhancement in apparent PTHR-binding affinity, as did the Glu<sup>19</sup> to Arg substitution, consistent with the potency-enhancing effects seen previously for this substitution in PTH(1-34) and PTH(1-20) peptides, when assessed in cAMP stimulation assays (Shimizu, M., *et al.* (2002) *Biochemistry* 41:13224-13233). Combining the Ala<sup>22</sup> substitution with either the Ala<sup>19</sup> or Arg<sup>19</sup> substitution did not lead to further improvements in PTHR-binding affinity (Table 1).

[00158] It was then assessed whether any of the alanine substitutions affected binding interactions to the receptor's J domain using PTHR-delNt. For these experiments, membranes prepared from COS-7 cells transiently transfected with PTHR-delNt were used, and, as a tracer radioligand, <sup>125</sup>I-[Aib<sup>1,3</sup>,M]PTH(1-15) (<sup>125</sup>I-[Aib<sup>1,3</sup>,Nle<sup>8</sup>,Gln<sup>10</sup>,homoarginine<sup>11</sup>,Ala<sup>12</sup>,Trp<sup>14</sup>,Tyr<sup>15</sup>]PTH(1-15)NH<sub>2</sub>), which binds exclusively to the J domain (Shimizu, N., *et al.* (2005) *J Biol Chem* 280:1797-807). To increase the total specific binding of <sup>125</sup>I-[Aib<sup>1,3</sup>,M]PTH(1-15) to these membranes, the cells were co-transfected with a negative-dominant Gα<sub>s</sub> mutant subunit, Gα<sub>s</sub>(α<sub>3</sub>β<sub>5</sub>,Gly<sup>226</sup>Ala,Ala<sup>366</sup>Ser); this Gα<sub>s</sub><sup>ND</sup> mutant is thought to couple to cognate receptors, and thus stabilize their high-affinity conformations, more efficiently than does wild-type Gα<sub>s</sub> (Berlot, C. H. (2002) *J Biol Chem* 277:21080-5). The apparent affinity observed for a PTH analog in these assays performed with PTHR-delNt and <sup>125</sup>I-

[Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub> thus reflects the overall strength of the interactions that occur between that ligand and the PTHR J domain.

[00159] The unlabeled [Aib<sup>1,3</sup>,M]PTH(1-15)NH<sub>2</sub> control peptide bound to these membranes with high apparent affinity, whereas unmodified PTH(1-31)NH<sub>2</sub> bound with much weaker affinity (IC<sub>50</sub>s = 2.2±0.5 nM and 3,700±400 nM, respectively, *P* = 0.001; FIG. 1C and D; Table 1). The weak binding that PTH(1-31)NH<sub>2</sub> exhibits on PTHR-delNt highlights the importance of the PTHR N domain in determining the overall affinity with which this non-optimized ligand binds to the wild-type PTHR. None of the alanine substitutions in the PTH(1-31)NH<sub>2</sub> scaffold strongly affected binding to PTHR-delNt. Most strikingly, the Arg<sup>20</sup> to Ala substitution, which abolished binding to the PTHR, caused only an approximate five-fold reduction in affinity for PTHR-delNt (FIG. 1A vs. C). Similarly, the Ala substitutions at Trp<sup>23</sup> and Leu<sup>24</sup>, which reduced affinity for the PTHR by 19- and 12-fold, respectively, reduced affinity for PTHR-delNt by only two-fold. These findings thus suggest that the mechanisms by which the Ala substitutions at Arg<sup>20</sup>, Trp<sup>23</sup> and Leu<sup>24</sup> impair binding to the intact PTHR are largely independent of ligand interactions to the PTHR J domain.

[00160] None of the substitutions had a major impact on the secondary structure of the peptide, as revealed by circular dichroism (CD) spectroscopy analysis. Thus, the CD spectrum of each analog exhibited clear negative deflections in the region of 209 nm and 222 nm, which are characteristic of  $\alpha$ -helical structure (FIG. 2). For PTH(1-31), these negative deflections have been shown by peptide fragment analysis to be derived largely from  $\alpha$ -helix in the (17-31) region (Neugebauer, W., *et al.* (1992) *Biochemistry* 31:2056-2063; Barbier, J. R., *et al.* (2005) *J Biol Chem.* 280:23771-23777). The number of helical residues per peptide chain, calculated from the CD signal at 222 nm, was between 7 and 10 for each of the peptides studied (Table 1). The lack of an effect of the substitutions on peptide secondary structure seen in these studies is consistent with the known helix-forming propensity of alanine (Chakrabarty, A., *Nature* 351:586-8).

Table 1: Helical Contents and PTHR-Binding Properties of PTH(1-31)NH<sub>2</sub> Analogs

	CD helical residues	PTHR-WT (HKRK-B7 cells) <i>IC</i> <sub>50</sub>	<i>n</i>	PTHR-delNT (COS-7) <i>IC</i> <sub>50</sub>	<i>n</i>
PTH(1-31)NH <sub>2</sub>	8	68 ± 10	23	3,666 ± 431	13
Ser <sup>17</sup> →Ala	10	57 ± 12	4	4,578 ± 386	4
Met <sup>18</sup> →Ala	10	92 ± 14	3	8,799 ± 2,334	4
Glu <sup>19</sup> →Ala	9	49 ± 11	4	2,940 ± 833	4
Arg <sup>20</sup> →Ala	10	>10,000	3	19,343 ± 8,100	4
Val <sup>21</sup> →Ala	10	200 ± 18	3	5,686 ± 486	4
Glu <sup>22</sup> →Ala	9	36 ± 2	4	5,857 ± 2,237	4
Trp <sup>23</sup> →Ala	7	1,312 ± 139	3	7,357 ± 1,832	4
Leu <sup>24</sup> →Ala	7	807 ± 165	3	8,764 ± 1,167	4
Arg <sup>25</sup> →Ala	7	281 ± 67	3	7,177 ± 1,083	4
Lys <sup>26</sup> →Ala	7	125 ± 8	3	6,210 ± 948	4
Lys <sup>27</sup> →Ala	9	217 ± 7	3	7,288 ± 615	4
Leu <sup>28</sup> →Ala	8	290 ± 63	3	3,459 ± 482	4
Gln <sup>29</sup> →Ala	9	44 ± 11	3	1,931 ± 303	4
Asp <sup>30</sup> →Ala	10	63 ± 7	4	1,545 ± 215	4
Val <sup>31</sup> →Ala	9	176 ± 23	4	4,911 ± 1,341	4
Glu <sup>19,22</sup> →Ala,Ala	9	49 ± 9	4	619 ± 75	4
Glu <sup>19,22</sup> →Arg,Ala	8	54 ± 13	4	360 ± 19	4
Glu <sup>19</sup> →Arg	8	42 ± 6	4	1,226 ± 332	4
Arg <sup>20</sup> →Gln	7	>10,000	3	10,284 ± 121	3
Arg <sup>20</sup> →Glu	7	>10,000	3	14,747 ± 324	3
Arg <sup>20</sup> →Lys	8	>10,000	3	7,105 ± 1,275	3
Arg <sup>20</sup> →Nle	12	15,404 ± 2,334	4	10,688 ± 1,033	3
Arg <sup>20</sup> →Cit	9	11,925 ± 2,151	4	10,624 ± 1,612	3
Arg <sup>20</sup> →Orn	6	>10,000	4	5,530 ± 279	3
Arg <sup>20</sup> →Apa	N.D.	>10,000	3	5,740 ± 775	3
Arg <sup>20</sup> →Gph	N.D.	>10,000	3	2,047 ± 442	3
Arg <sup>20</sup> →PipGly	8	14,135 ± 5,345	4	6,605 ± 2,407	5
[Aib <sup>1,3</sup> ,M]PTH(1-15)	N.D.	N.D.		2.2 ± 0.5	8

---

Substitutions were introduced into hPTH(1-31)NH<sub>2</sub>. The helical residue values were calculated from  $[\theta]_{222}$ , the mean-residue ellipticities observed in the CD spectra at 222 nm. Competition binding studies were performed in intact HKRK-B7 cells stably transfected with the hPTHr using <sup>125</sup>I-[Nle<sup>8,21</sup>,Tyr<sup>34</sup>]ratPTH(1-34)NH<sub>2</sub> tracer radioligand, and in membranes prepared from COS-7 cells transiently transfected with PTHR-delNt and a negative-dominant G<sub>αs</sub>, using <sup>125</sup>I-[Aib<sup>1,3</sup>,Nle<sup>8</sup>,Gln<sup>10</sup>,Har<sup>11</sup>,Ala<sup>12</sup>,Trp<sup>14</sup>,Tyr<sup>15</sup>]ratPTH(1-15)NH<sub>2</sub> tracer radioligand. Binding values (nM) are means (+/- s.e.m.) of data from the number of experiments indicated (n).

## EXAMPLE 2

[00161] *Substitutions with cyclohexylalanine* - The data presented here, and previously (Gardella, T. J., *et al.* (1993) *Endocrinology* 132:2024-2030; Oldenburg, K. R., *et al.* (1996) *Journal Of Biological Chemistry* 271:17582-17591; Reidhaar-Olson, J., *et al.* (2000) *Mol Cell Endocrinol* 160:135-147.) suggest that hydrophobicity in the PTH(17-31) region, as provided principally by the side chains of Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup>, is a physicochemical property of the domain that is key for high affinity binding to the receptor. To evaluate further the general importance of hydrophobicity per se in this region of the ligand, each residue in the PTH(17-31) region was substituted with cyclohexylalanine (Cha), a non-encoded amino acid analog that would provide bulk side chain hydrophobicity at the targeted site while altering the chemical and topological features of the side chain, and the effects of the substitutions on binding to the PTHR and to PTHR-delNt were assessed. In these studies, [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> was used as a scaffold peptide, in order to augment the capacity to assess binding to PTHR-delNt, since the Glu<sup>19</sup> to Arg and Ser<sup>1</sup> to Ala substitutions were known to improve interaction with the truncated receptor (Shimizu, M., *et al.* (2002) *Biochemistry* 41:13224-13233; Shimizu, M., *et al.* (2000) *J. Biol. Chem.* 275:21836-21843).

[00162] CD analyses indicated that none of the Cha substitutions disrupted the α-helical content of the peptide, although the helical deflections at 209 and 222 nm were enhanced for the Cha<sup>20</sup> and Cha<sup>27</sup> analogs, resulting in helical content values of 16 and 18 residues, respectively, versus eight residues for the

parent peptide (FIG. 8; Table 2). The basis for these more intense CD deflections with the Cha<sup>20</sup> and Cha<sup>27</sup> analogs, which were not accompanied by parallel changes in functional activity, is not clear at present.

[00163] The effects of the Cha substitutions on binding to the intact PTHR in HKRK-B7 cells were generally milder than those of the alanine substitutions. The Cha substitution at Arg<sup>20</sup> reduced apparent binding affinity by 120-fold ( $P = 0.005$ ), and those at Trp<sup>23</sup> and Leu<sup>24</sup> reduced affinity by 14- and 11-fold, respectively ( $P < 0.002$ ; FIGS. 3A and B; Table 2). Although mild, the effects of the Cha substitutions at Trp<sup>23</sup> and Leu<sup>24</sup> suggest that hydrophobicity per se is not the main structural feature of these two side chains that determines their contribution to PTHR binding affinity. The Cha substitutions at the remaining positions altered PTHR-binding affinity by only six-fold or less.

[00164] On PTHR-delNt, the Cha substitutions at Arg<sup>25</sup> and Lys<sup>26</sup> reduced binding affinity by approximately five- to seven-fold; the remaining Cha substitutions, including those at Arg<sup>20</sup>, Trp<sup>23</sup> and Leu<sup>24</sup>, altered affinity for PTHR-delNt by three-fold or less (FIGS. 3C and D; Table 2). These results with Cha substitutions are in close agreement with those obtained with the corresponding Ala substitutions, and suggest that the detrimental effects that the Cha substitutions at positions 20, 23 and 24 have on PTHR-binding affinity are not due to altered interactions with the PTHR J domain.

Table 2 Helical Contents and PTHR-Binding Properties of [Ala<sup>1</sup>,Arg<sup>19</sup>]hPTH(1-31)NH<sub>2</sub> Analogs.

	CD helical residues	PTHr-WT (HKRK-B7 cells) IC <sub>50</sub>	<i>n</i>	PTHr-delNT (COS-7) IC <sub>50</sub>	<i>n</i>
[Ala <sup>1</sup> ,Arg <sup>19</sup> ]PTH(1-31)NH <sub>2</sub>	8	38 ± 8	7	397 ± 40	4
Ser <sup>17</sup> →Cha	9	112 ± 13	3	179 ± 41	4
Met <sup>18</sup> →Cha	8	87 ± 13	3	408 ± 32	4
Arg <sup>19</sup> →Cha	10	233 ± 57	3	780 ± 83	4
Arg <sup>20</sup> →Cha	16	4,738 ± 333	3	632 ± 79	4
Val <sup>21</sup> →Cha	9	74 ± 15	3	564 ± 172	4
Glu <sup>22</sup> →Cha	8	75 ± 9	3	126 ± 45	4
Trp <sup>23</sup> →Cha	8	546 ± 70	3	867 ± 402	4
Leu <sup>24</sup> →Cha	9	436 ± 10	3	283 ± 68	4
Arg <sup>25</sup> →Cha	9	103 ± 27	3	2711 ± 1306	4
Lys <sup>26</sup> →Cha	6	172 ± 27	3	1872 ± 281	4
Lys <sup>27</sup> →Cha	18	91 ± 17	3	629 ± 72	4
Leu <sup>28</sup> →Cha	8	65 ± 10	3	226 ± 39	4
Gln <sup>29</sup> →Cha	8	61 ± 14	3	319 ± 57	4
Asp <sup>30</sup> →Cha	8	54 ± 7	3	305 ± 126	4
Val <sup>31</sup> →Cha	9	66 ± 20	3	466 ± 298	4
Arg <sup>19</sup> →Glu	9	125 ± 29	4	18,856 ± 9649	5
Arg <sup>20</sup> →Glu	8	>10,000	3	776 ± 380	5
Val <sup>21</sup> →Glu	7	447 ± 172	4	1,615 ± 516	5
Trp <sup>23</sup> →Glu	7	6,044 ± 2,636	3	434 ± 84	5
Leu <sup>24</sup> →Glu	6	>10,000	3	1,041 ± 197	5
Arg <sup>25</sup> →Glu	5	189 ± 53	4	1,629 ± 274	5
Lys <sup>26</sup> →Glu	6	60 ± 19	3	832 ± 215	5
Lys <sup>27</sup> →Glu	5	156 ± 50	3	1,266 ± 293	5
Leu <sup>28</sup> →Glu	5	10,360 ± 3,983	3	974 ± 252	5

Cyclohexylalanine and glutamate substitutions were introduced into [Ala<sup>1</sup>,Arg<sup>19</sup>]hPTH(1-31)NH<sub>2</sub>. The helical residue values were calculated from  $[\theta]_{222}$ , the mean-residue ellipticities observed in the CD spectra at 222 nm. Competition binding studies were performed in intact HKRK-B7 cells stably transfected with the hPTHr using <sup>125</sup>I-[Nle<sup>8,21</sup>,Tyr<sup>34</sup>]rPTH(1-34)NH<sub>2</sub> tracer radioligand, and in membranes prepared from COS-7 cells transiently transfected with hPTHr-delNT and a negative-dominant Gas using <sup>125</sup>I-[Aib<sup>1,3</sup>,Nle<sup>8</sup>,Gln<sup>10</sup>,Har<sup>11</sup>,Ala<sup>12</sup>,Trp<sup>14</sup>,Tyr<sup>15</sup>]rPTH(1-15)NH<sub>2</sub> tracer radioligand. Binding values (nM) are means (+/- s.e.m.) of data from the number of experiments indicated (*n*).

## EXAMPLE 3

[00165] *Non-conservative Glu substitutions* - Substitutions were introduced that would more strongly disrupt binding to the intact PTHR, but only marginally impact binding to PTHR-delNt. To do this, glutamic acid was introduced, as non-conservative single substitutions, at each position in the carboxyl-terminal segment of [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> that was otherwise occupied by a hydrophobic or positively charged amino acid. The analogs were again assessed for binding to the intact PTHR and to PTHR-delNt. Several of the Glu substitutions caused severe losses of binding affinity for the intact PTHR. Thus, Glu substitution at Arg<sup>20</sup> and Leu<sup>24</sup> abolished detectable binding and those at Leu<sup>28</sup>, Trp<sup>23</sup> and Val<sup>21</sup> reduced apparent affinity by 270-160- and 12-fold, respectively (FIGS. 4A and B; Table 2). Modest, two-to five-fold reductions in PTHR-binding affinity occurred with the Glu substitutions at Arg<sup>19</sup>, Arg<sup>25</sup>, Lys<sup>26</sup> and Lys<sup>27</sup>. None of the Glu substitutions substantially altered the CD profile of the peptide (Table 2).

[00166] Each of the Glu substitutions had only a mild impact on binding to PTHR-delNt. Thus, Glu substitutions at Arg<sup>20</sup>, Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup>, which reduced apparent affinity for the PTHR by 150-fold or more, reduced affinity for the truncated PTHR by less than three-fold (FIGS. 4C and D; Table 2). The Glu substitutions at Val<sup>21</sup> and Arg<sup>25</sup> each reduced affinity for PTHR-delNt by about four-fold ( $P < 0.01$ ); which were similar to the effects that the same substitutions had on binding to the intact PTHR. The Arg<sup>19</sup> to Glu substitution reduced affinity on PTHR-delNt by 50-fold ( $IC_{50} = 19,00 \pm 10,00$  nM, versus  $400 \pm 40$  nM). This deleterious effect is fully consistent with the enhancements in cAMP-stimulating potency seen for the reciprocal Glu<sup>19</sup> to Arg substitution in PTH(1-20) and PTH(1-34) analogs in COS-7 cells expressing PTHR-delNt (Shimizu, M., *et al.* (2002) *Biochemistry* 41:13224-13233).

## EXAMPLE 4

[00167] *Analysis of arginine-20* - Arginine-20, one of the most conserved residues in PTH and PTHrP ligands, has been shown by a number of studies to be critical determinant of the PTHR-interaction mechanism (Oldenburg, K. *et al.* (1996) *Journal Of Biological Chemistry* 271:17582-17591; Barbier, J., *et al.* (2001) *Biochemistry* 40:8955-8961; Reidhaar-Olson, J., *et al.* (2000) *Mol Cell Endocrinol* 160:135-147), but a mechanistic role for this residue has yet to be described. Barbier *et al.* analyzed the effects of 11 different amino acid analog substitutions at this position in PTH(1-31)NH<sub>2</sub> on the cAMP-stimulating potency of the peptide in ROS 17/2.8 cells, and found that none of the substitutions preserved potency. Thus even the close arginine homologs: citrulline, 4-piperidyl-(N-amidino)glycine (PipGly) and 4-piperidyl-(N-amidino)alanine (PipAla), reduced potency 8-, 5- and >21-fold, respectively, and lysine abolished activity altogether (Barbier, J., *et al.* (2001) *Biochemistry* 40:8955-8961). To further dissect the functional role of Arg<sup>20</sup>, the same position-20 modified PTH(1-31)NH<sub>2</sub> analogs were examined for their capacities to bind to the intact PTHR and to PTHR-delNt.

[00168] The replacement of Arg<sup>20</sup> by either Gln, Glu, Lys, (S)-2-amino-4[(2-amino)pyrimidinyl]methylalanine (Apa) or L-4-guanidinoPhenylalanine (Gph), resulted in a complete loss of detectable binding affinity for the intact PTHR (FIGS. 5A and B; Table 1). The substitutions of PipGly, norleucine and citrulline reduced apparent PTHR-binding affinity by about 200-fold, relative to that of the parent peptide. Each of the position 20 substitutions had relatively mild effects on binding to PTHR-delNt, as the IC<sub>50</sub> of each of analog was within about four-fold of that of the parent PTH(1-31)NH<sub>2</sub> peptide (FIGS. 5C and D; Table 1). These effects are consistent with those observed for the Ala-, Cha- and Glu substitutions at position 20, as they indicate that the effects of the substitutions on binding are largely independent of interactions to the PTHR J domain. The Arg<sup>20</sup> to Glu substitution in PTH(1-31)NH<sub>2</sub> resulted in a small, approximately four-fold reduction in apparent binding



affinity for PTHR-delNt, suggesting that changes at this position can have an influence, direct or indirect, on ligand interactions that occur to the PTHR J domain.

#### EXAMPLE 5

[00169] *Effects on cAMP and IP signaling* - Selected analogs of [Ala<sup>1</sup>,Arg<sup>19</sup>]hPTH(1-31)NH<sub>2</sub> with substitutions that markedly impaired binding to HKRK-B7 cells were assessed for their capacity to bind to the PTHR in ROS 17/2.8 cells and to stimulate cAMP-signaling responses in these cells. These bone-derived cells endogenously express the rat PTHR at a more physiological level than is found in HKRK-B7 cells (approximately 80,000 PTHRs per cell vs. 950,000 PTHRs per cell) and, have generally been found (data not shown) to yield closer correlations between PTH analog binding affinity and cAMP-signaling potency than do HKRK-B7 cells, presumably due to their lower PTHR expression level. Competition binding assays performed in these cells utilizing <sup>125</sup>I-PTH(1-34) tracer radioligand revealed effects of the substitutions on PTHR-binding affinity that paralleled the corresponding effects seen in HKRK-B7 cells. Thus, in ROS 17/2.8 cells, the Glu substitutions at Arg<sup>20</sup>, Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup> reduced affinity by 20,000- to 150-fold, and the Cha substitutions at Arg<sup>20</sup>, Trp<sup>23</sup> and Leu<sup>24</sup> reduced affinity by 10- to 120-fold (FIG. 6A; Table 3). These effects on binding affinity in ROS 17/2.8 cell were accompanied by parallel reductions in cAMP-stimulating potency, although the effects on cAMP signaling potency were generally not as severe as the corresponding effects on binding affinity (FIG. 6B; Table 3). Each substituted analog also produced the same maximum cAMP response as did the parental peptide. Each of the alanine - substituted PTH(1-31)NH<sub>2</sub> analogs was also tested at a concentration of 1x10<sup>-6</sup> M, for the capacity to stimulate inositol phosphate (IP) production in COS-7 cells transfected with the PTHR (PTH-induced IP responses in HKRKB7 and ROS 17/2.8 cells are generally too low to detect, data not shown). Each

analog stimulated the same four-fold increase in total IP levels as did the parent peptide. Thus, the data combined indicate that the substitutions that caused strong effects on PTHR-binding affinity did not cause severe disruptions in either the cAMP or inositol phosphate signaling capacity of the ligand. This finding is consistent with the notion that the principal ligand determinants of receptor activation, in terms of  $G\alpha_s$ -mediated cAMP and  $G\alpha_q$ -mediated  $IP_3$  signaling, reside in the amino-terminal domain of the ligand (Shimizu, M., *et al.* (2000) *J. Biol. Chem.* 275:21836-21843; Takasu, H., *et al.* (1999) *J Bone Miner Res.* 14:11-20) and are not perturbed by the carboxyl-terminal substitutions of the PTH(1-31) analogs.

Table 3: Activities in ROS 17/2.8 Cells

	Binding <i>IC</i> <sub>50</sub>	cAMP <i>EC</i> <sub>50</sub>
[Ala <sup>1</sup> ,Arg <sup>19</sup> ]PTH(1-31)	1.6 ± 0.2	0.78 ± 0.24
Arg <sup>20</sup> →Cha	290 ± 130	7.5 ± 3.0
Trp <sup>23</sup> →Cha	19 ± 7	0.82 ± 0.11
Leu <sup>24</sup> →Cha	7.3 ± 0.8	0.83 ± 0.17
Arg <sup>20</sup> →Glu	5,800 ± 1,200	59 ± 31
Trp <sup>23</sup> →Glu	220 ± 60	4.0 ± 2.0
Leu <sup>24</sup> →Glu	29,000 ± 5,000	250 ± 70
Leu <sup>28</sup> →glu	3,100 ± 2,100	11 ± 2

Peptide are analogs of [Ala<sup>1</sup>,Arg<sup>19</sup>]hPTH(1-31)NH<sub>2</sub>. Competition binding was assessed in cells using <sup>125</sup>I-[Nle<sup>8,21</sup>,Tyr<sup>34</sup>]rPTH(1-34)NH<sub>2</sub> tracer radioligand. Values (nM) are means (+/- s.e.m.) of data from three experiments, each performed in duplicate.

## CONCLUSIONS

**[00170]** There is currently little information available that speaks to the specific functional roles that amino acid side chains in the (17-31) domain of PTH, the principal-receptor binding domain, play in the receptor-interaction process. The current studies were designed to explore these roles using a number of new PTH(1-31) analogs modified in the (17-31) domain, together with a new PTH-receptor binding assay that utilizes PTHR-delNt as a means to resolve whether a ligand substitution alters interaction to the receptor's amino-terminal

extracellular (N) domain, or to its juxtamembrane (J) domain containing the extracellular loops and transmembrane helices. The overall data from these studies are largely consistent with the two-domain model that has been proposed for the PTH-PTHr interaction mechanism, as well as for the class 2 GPCRs in general, in that they suggest that the major binding interactions occur between residues in the carboxyl-terminal portion of the ligand and the N domain of the receptor.

**[00171]** In the studies on a large number of PTH(1-31)NH<sub>2</sub> or [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> analogs altered in the (17-31) region, it was found that the strongest effects on binding to the intact PTHr occurred with non-conservative Glu substitutions at Arg<sup>20</sup>, Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup>-- each of which reduced PTHr-binding affinity by at least 150-fold. These data are thus in agreement with previous PTH-substitution studies which highlighted the importance of these same residues in binding to the intact PTHr (Gardella, T. J., *et al.* (1993) *Endocrinology* 132:2024-2030; Oldenburg, K. R., *et al.* (1996) *Journal Of Biological Chemistry* 271:17582-17591; Reidhaar-Olson, J., *et al.* (2000) *Mol Cell Endocrinol* 160:135-147; Neugebauer, W., *et al.* (1992) *Biochemistry* 31:2056-2063). As is now shown that the same Glu substitutions have little or no effect on binding to PTHr-delNt, and do not cause major perturbations of the ligand's helical structure, it can be concluded that the side chains of Arg<sup>20</sup>, Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup> indeed contribute to the PTHr-binding process by mechanisms that largely, if not completely, involve interactions to the receptor's N-terminal domain.

**[00172]** The side chains of Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup> form the hydrophobic face of the amphiphilic  $\alpha$ -helix predicted for the (17-31) region of PTH (Neugebauer, W., *et al.* (1992) *Biochemistry* 31:2056-2063; Epand, R. E. (1983) *Mol and Cell Biol* 57:41-47). It has been suggested for peptide ligands in general (Sargent, D., and Schwyzer, R. (1986) *Proc Natl Acad Sci USA* 83:5774-5778), including for PTH (Pellegrini, M., *et al.* (1998) *Biochemistry* 37:12737-12743), that such an amphiphilic  $\alpha$ -helix can contribute to the receptor-binding process by enabling nonspecific hydrophobic interactions with the lipid

component of the cell membrane, to thus facilitate a two-dimensional diffusion to the receptor. That the Glu substitutions at Trp<sup>23</sup>, Lcu<sup>24</sup> and Leu<sup>28</sup> only mildly impaired binding to PTHR-delNt strongly suggest, however, that the effects of these substitutions are not based on a disruption of nonspecific interactions with the cell membrane, as such effects would have been equally apparent on the intact PTHR and on PTHR-delNt. This conclusion is further supported by findings that the replacement of Trp<sup>23</sup> and Leu<sup>24</sup> by cyclohexylalanine (Cha), which preserves hydrophobicity and thus the capacity for non-specific interactions with the lipid membrane, reduced binding on the intact PTHR by approximately 12-fold, and again had little or no effect on binding to PTHR-delNt (Table 2). It thus appears that more specific physicochemical features of the tryptophan-23 and leucine-24 side chains, other than hydrophobicity per se, are required for high affinity interactions with the receptor.

[00173] It is clear from the current data, and that presented elsewhere (Oldenburg, K. R., *et al.* (1996) *Journal Of Biological Chemistry* 271:17582-17591; Barbier, J., *et al.* (2001) *Biochemistry* 40:8955-8961; Reidhaar-Olson, J., *et al.* (2000) *Mol Cell Endocrinol* 160:135-147), that the highly conserved arginine at position 20 of PTH plays a key role in the PTHR-binding process. Of ten substitutions tested at this position in the PTH(1-31)NH<sub>2</sub> scaffold peptide, citrulline, PipGly and norleucine reduced affinity for the PTHR by -200-fold and the remaining substitutions abolished binding altogether. As each of these substitutions had only a minor effect on binding to PTHR-delNt, the side chain of Arg<sup>20</sup>, like those of Trp<sup>23</sup>, Leu<sup>24</sup> and Leu<sup>28</sup>, must contribute to the PTHR-binding process via a mechanism that primarily involves interactions to the PTHR N domain. The overall data, however, based on a considerable number of structurally varied amino acids, are consistent with the notion that multiple components of the arginine side chain, including the cationic and H-bonding nitrogen atoms of the guanidino group and the aliphatic side chain linker, participate in the interaction and must align precisely with cognate functional groups in the receptor (Barbier,

J., *et al.* (2001) *Biochemistry* 40:8955-8961). In any event, it now seems clear that these cognate functional groups are located predominantly, if not exclusively, in the receptor's N domain region.

[00174] In contrast to arginine 20, residue 19 of PTH appears to interact predominantly with the PTHR J domain. This can be seen in the current data by the 90-fold reduction in binding affinity on PTHR-delNt caused by the Arg<sup>19</sup> to Glu substitution in [Ala<sup>1</sup>,Arg<sup>19</sup>]PTH(1-31)NH<sub>2</sub> -- the strongest effect on binding to PTHR-delNt of any substitution tested. This reduction in binding affinity mirrors the enhancing effect that the Glu<sup>19</sup> to Arg substitution in PTH(1-34) and PTH(1-20) analogs has on the cAMP-stimulating potencies of the analogs in COS-7 cells expressing PTHR-delNt (Shimizu, M., *et al* (2002) *Biochemistry* 41:13224-13233). That the side chain of residue 19 comes within proximity of the PTHR J domain is further shown by the cross-linking of [Bpa<sup>19</sup>]PTHrP(1-36) and [Bpa<sup>19</sup>]PTH(1-20) analogs to the extracellular end of transmembrane helix 2 of the PTHR (Gensure, R. C., *et al* (2003) *Mol Endocrinol* 17:2647-58). These data for residue 19, considered together with those described above for Arg<sup>20</sup>, intriguingly suggest that the 19/20 position in the ligand may comprise a boundary between segments of PTH that interact with the N and J domain regions of the receptor — PTH segments (20-31) and (1-19), respectively.

[00175] Finally, the studies provide insights into the topology of the PTH ligand, as it is bound to the receptor, and of the spatial relationship of the N and J domains of the ligand-occupied PTHR. Subtle but consistent effects of substitutions at positions 21, 25, 26 and 27 on interaction of the ligand with PTHR-delNt were found, and these effects were accompanied by approximately proportional effects on interaction with the intact PTHR. Such findings point to the possibility that the side chains of these residues, while not contributing in a major way to overall binding energy, can influence the ligand-binding interactions that occur to the PTHR J domain. The cationic side chains of Arg<sup>25</sup>, Lys<sup>26</sup> and Lys<sup>27</sup> would form the hydrophilic face of the ligand's amphiphilic  $\alpha$ -helix, and Val<sup>21</sup> would lie at the edge of this face

(Barbier, J. R., *et al.* (2005) *J Biol Chem.* 280:23771-23777). It is possible that these side chains modulate binding affinity indirectly, for example, by interacting with the phospholipid head groups of the cell membrane bilayer, to facilitate diffusion to the receptor, as discussed above. However, more direct interactions with presumably anionic and/or hydrophobic groups in the extracellular loops and/or transmembrane domain regions of the receptor are also possible. Indeed, the cross-linking of [Lys<sup>27</sup>(Bp<sub>z</sub>)]PTH(1-34) to the first extracellular loop of the PTHR (Greenberg, Z., *et al.* (2000) *Biochemistry* 39:8142-8152) supports this latter possibility, as do the recent findings that backbone methylations at Ser<sup>17</sup>, Trp<sup>23</sup> and Lys<sup>26</sup> in PTH(1-31)NH<sub>2</sub> impair, albeit modestly, the capacity of the ligand to stimulate cAMP signaling via PTHR-delNt (Barbier, J. R., *et al.* (2005) *J Biol Chem.* 280:23771-23777).

[00176] If the ligand's carboxyl-terminal  $\alpha$ -helical domain, in addition to its amino-terminal PTH(1-19) domain (see above) interact with the PTHR J domain, then a fold or bend between the amino - and carboxyl-terminal domains of the bound ligand would seem to be required in order to accommodate both sets of interactions. Most solution-phase NMR studies of PTH and PTHrP ligands indeed show a hinge or flexible region between the amino-terminal and carboxyl-terminal domains (Chen, Z., *et al.* (2000) *Biochemistry* 39:12766-12777; Pellegrini, M., *et al.* (1998) *J. Biol. Chem.* 273:10420-10427; Barden, J. A., and Kemp, B. E. (1994) *Biochim Biophys Acta* 1208:256-262; Peggion, E., *et al.* (2002) *Biochemistry* 41:8162-8175), that could facilitate such a bend. That the receptor-bound ligand adopts a mid-region fold is further suggested by the finding that [Bpa<sup>11</sup>]PTH(1-34) and [Bpa<sup>21</sup>]PTH(1-34) analogs each cross-link to the same segment (Ala<sup>165</sup>-Asn<sup>176</sup>) of the PTHR N domain, as the two contacts are not easily reconciled with a linear ligand structure (Wittelsberger, A., *et al.* (2006) *Biochemistry* 45:2027-34). The notion that PTH adopts a mid-region bend in the receptor bound state differs from a recent model of the pituitary adenylyl cyclase-activating peptide bound to its related class 2 GPCR, since in this model the ligand is portrayed as a linear extended helix

making extensive contacts to the receptor's N domain (Tan, Y. V., *et al.* (2006) *J Biol Chem* 281:12792-8).

[00177] If the carboxyl-terminal helical domain of PTH indeed interacts with J domain, as well as the N domain regions of the receptor, via its hydrophobic and hydrophilic faces, respectively, and these interactions were to occur simultaneously, then the N and J domains of the occupied receptor would have to be near each other in order to accommodate both sets of interactions. This possibility is supported by the cross-linking of [Bpa<sup>27</sup>]PTH(1-34), having Lys<sup>27</sup> replaced by benzoylphenylalanine, to the receptor's N domain (Gensure, R., *et al.* (2001) *J Biol Chem* 276:28650-28658), and that of [Lys<sup>27</sup>(Bpz)]PTH(1-34) to the receptor's first extracellular loop (Greenberg, Z., *et al.* (2000) *Biochemistry* 39:8142-8152).

[00178] While various embodiments of the present invention have been described above, it should be understood that they have been presented by way of example only, and not limitation. It will be apparent to persons skilled in the relevant art that various changes in form and detail can be made therein without departing from the spirit and scope of the invention. Thus, the breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents. All publications, patents and patent applications cited herein are incorporated by reference in their entirety into the disclosure.

## WHAT IS CLAIMED IS:

1. A biologically active peptide at least 90% identical to a peptide consisting essentially of the formula:

(a)  $X_{01}$  ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSer  
Met $X_{02}$  $X_{03}$  $X_{04}$ Glu $X_{05}$  $X_{06}$ ArgLysLys $X_{07}$ GlnAspVal (SEQ ID NO:1);

(b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof;

wherein:

$X_{01}$  is Ser or Ala;

$X_{02}$  is Glu or Arg;

$X_{03}$  is Ala, Glu, Gln or Cha;

$X_{04}$  is Val or Glu;

$X_{05}$  is Trp, Ala, Glu or Cha;

$X_{06}$  is Leu, Ala, Glu or Cha; and

$X_{07}$  is Leu or Glu;

provided that said peptide is not hPTH(1-31)NH<sub>2</sub>.

2. A biologically active peptide consisting essentially of the formula:

(a)  $X_{01}$  ValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSer  
Met $X_{02}$  $X_{03}$  $X_{04}$ Glu $X_{05}$  $X_{06}$ ArgLysLys $X_{07}$ GlnAspVal (SEQ ID NO:1);

(b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof;

wherein:

$X_{01}$  is Ser or Ala;

$X_{02}$  is Glu or Arg;

$X_{03}$  is Ala, Glu, Gln or Cha;



X<sub>04</sub> is Val or Glu;

X<sub>05</sub> is Trp, Ala, Glu or Cha;

X<sub>06</sub> is Leu, Ala, Glu or Cha; and

X<sub>07</sub> is Leu or Glu;

provided that said peptide is not hPTH(1-31)NH<sub>2</sub>.

3. The peptide of claim 1, consisting essentially of the formula:

- (a) SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsn  
SerMetGluAlaValGluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:2);
- (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
- (c) pharmaceutically acceptable salts thereof; or
- (d) N- or C- derivatives thereof.

4. The peptide of claim 1, consisting essentially of the formula:

- (a) SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsn  
SerMetGluArgValGluAlaLeuArgLysLysLeuGlnAspVal (SEQ ID NO:3);
- (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
- (c) pharmaceutically acceptable salts thereof; or
- (d) N- or C- derivatives thereof.

5. The peptide of claim 1, consisting essentially of the formula:

- (a) SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsn  
SerMetGluArgValGluTrpAlaArgLysLysLeuGlnAspVal (SEQ ID NO:4);
- (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
- (c) pharmaceutically acceptable salts thereof; or
- (d) N- or C- derivatives thereof.

6. The peptide of claim 1, consisting essentially of the formula:
- (a) SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetGluGluValGluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:5);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or
  - (d) N- or C- derivatives thereof.
7. The peptide of claim 1, consisting essentially of the formula:
- (a) AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgValGluGluLeuArgLysLysLeuGlnAspVal (SEQ ID NO:6);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or
  - (d) N- or C- derivatives thereof.
8. The peptide of claim 1, consisting essentially of the formula:
- (a) AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgValGluTrpGluArgLysLysLeuGlnAspVal (SEQ ID NO:7);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or
  - (d) N- or C- derivatives thereof.
9. The peptide of claim 1, consisting essentially of the formula:
- (a) AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgValGluTrpLeuArgLysLysGluGlnAspVal (SEQ ID NO:8);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or
  - (d) N- or C- derivatives thereof.

10. The peptide of claim 1, consisting essentially of the formula:
- (a) AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgGluGluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:9);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or
  - (d) N- or C- derivatives thereof.
11. The peptide of claim 1, consisting essentially of the formula:
- (a) AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgChaValGluTrpLeuArgLysLysLeuGlnAspVal (SEQ ID NO:10);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or
  - (d) N- or C- derivatives thereof.
12. The peptide of claim 1, consisting essentially of the formula:
- (a) AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgValGluChaLeuArgLysLysLeuGlnAspVal (SEQ ID NO:11);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or
  - (d) N- or C- derivatives thereof.
13. The peptide of claim 1, consisting essentially of the formula:
- (a) AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetArgArgValGluTrpChaArgLysLysLeuGlnAspVal (SEQ ID NO:12);
  - (b) fragments thereof, containing amino acids 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29 or 1-30;
  - (c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

14. The peptide of claim 1, wherein said peptide is labeled with a label selected from the group consisting of radiolabel, fluorescent label, bioluminescent label, and chemiluminescent label.
15. A pharmaceutical composition comprising the peptide of claim 1 and a pharmaceutically acceptable carrier.
16. A nucleic acid molecule consisting essentially of a polynucleotide encoding the peptide of claim 1.
17. A recombinant vector comprising the nucleic acid molecule of claim 16.
18. A recombinant DNA molecule comprising: (1) an expression control region, said region in operable linkage with (2) a polynucleotide sequence coding for the peptide of claim 1.
19. A method of preparing a biologically active peptide comprising introducing into a host cell the recombinant DNA molecule of claim 18, and causing expression of said molecule.
20. A method of making a recombinant vector comprising inserting a nucleic acid molecule of claim 16 into a vector.
21. The recombinant DNA molecule of claim 18, wherein said control region includes a bacterial, viral, fungal or mammalian promoter.
22. A prokaryotic or eukaryotic host cell containing the recombinant DNA molecule of claim 18.

23. The cell of claim 22 which is bacterial.
24. A method for treating mammalian conditions characterized by decreases in bone mass, wherein said method comprises administering to a subject in need thereof an effective bone mass-increasing amount of the peptide of claim 1 and a pharmaceutically acceptable carrier.
25. The method of claim 24, wherein said effective bone mass-increasing amount of said peptide is administered by providing to the patient DNA encoding said peptide and expressing said peptide in vivo.
26. The method of claim 24, wherein said condition to be treated is osteoporosis.
27. The method of claim 24, wherein said condition to be treated is old age osteoporosis.
28. The method of claim 24, wherein said condition to be treated is menopausal osteoporosis.
29. The method of claim 24, wherein the effective amount of said peptide for increasing bone mass is from about 0.01  $\mu\text{g/kg/day}$  to about 1.0  $\mu\text{g/kg/day}$ .
30. The method of claim 24, wherein the method of administration is parenteral.
31. The method of claim 24, wherein the method of administration is subcutaneous.

32. The method of claim 24, wherein the method of administration is nasal insufflation.

33. A method for determining rates of bone formation, bone resorption and/or bone remodeling comprising administering to a patient an effective amount of a peptide of claim 1 and determining the uptake of said peptide into the bone of said patient.

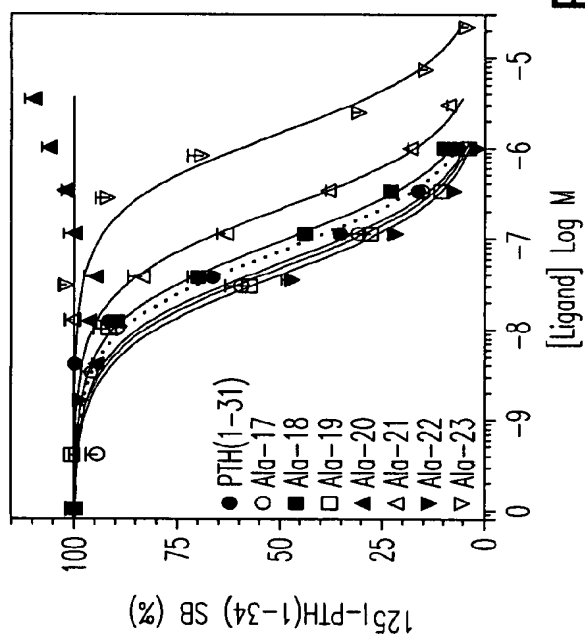


FIG. 1A

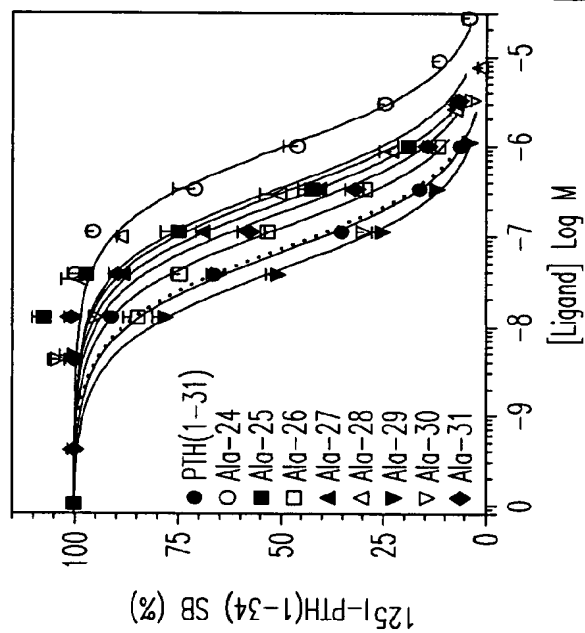


FIG. 1B

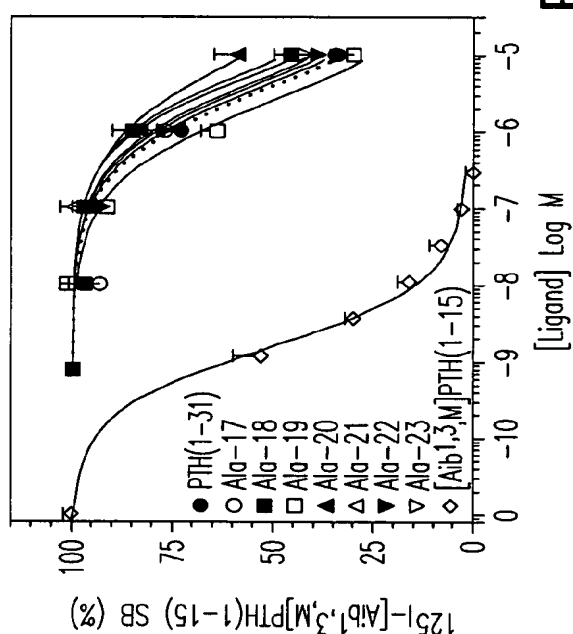


FIG. 1C

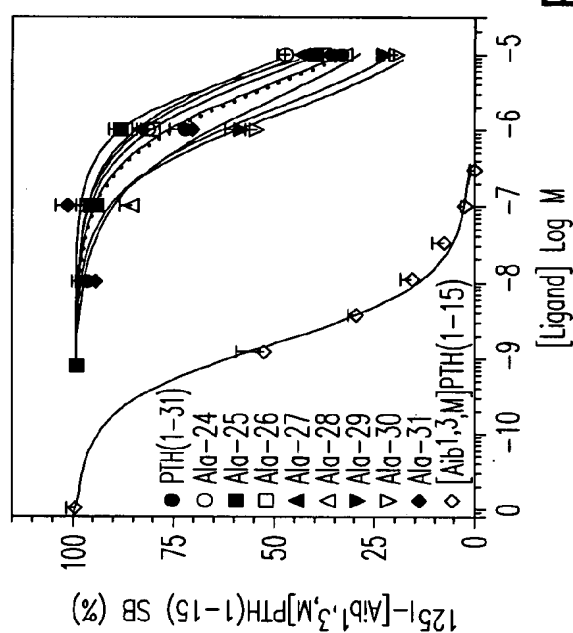


FIG. 1D

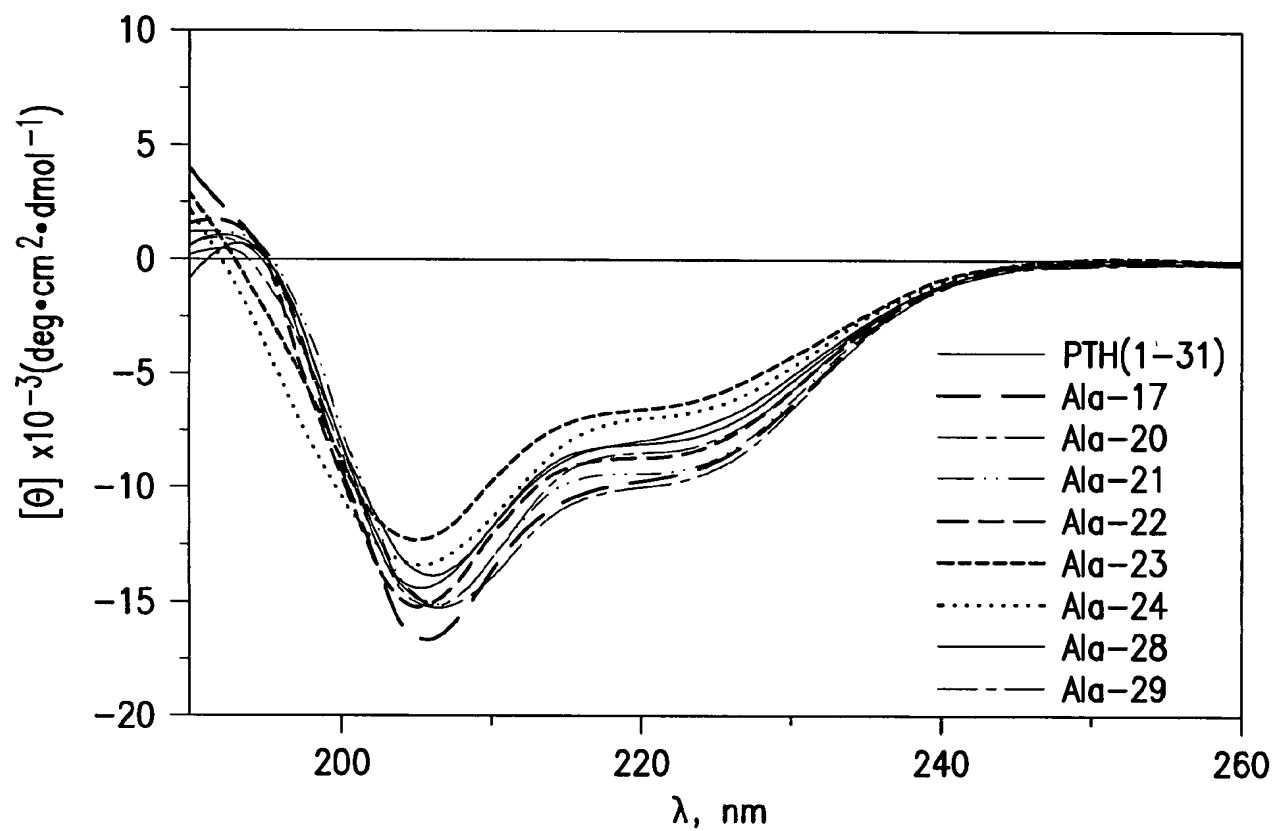


FIG.2



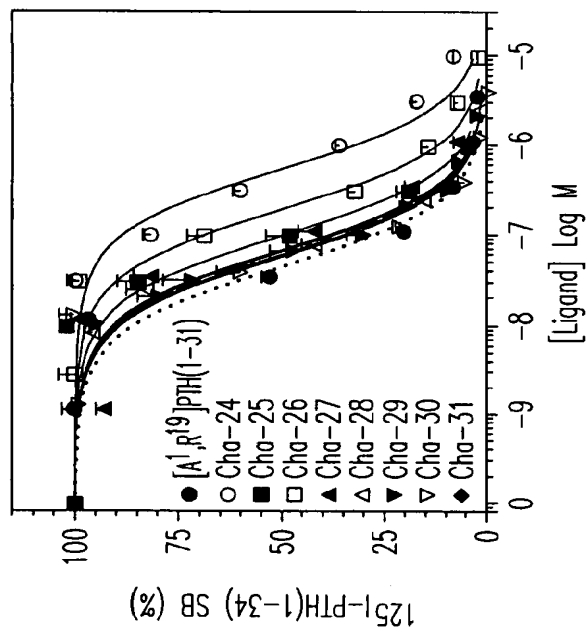


FIG. 3B

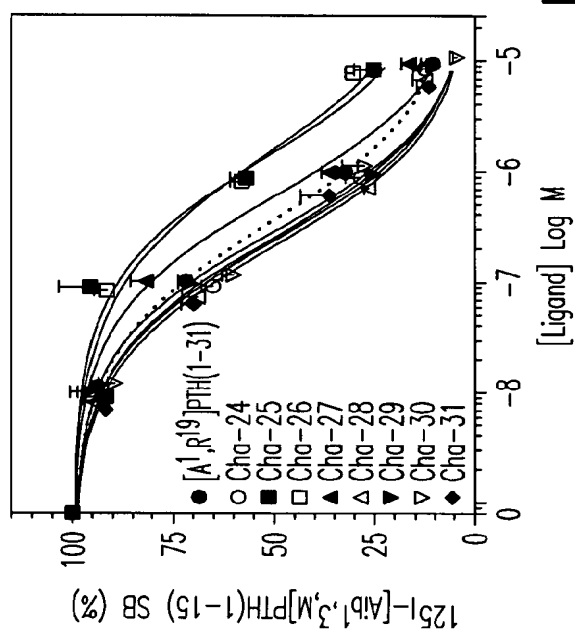


FIG. 3D

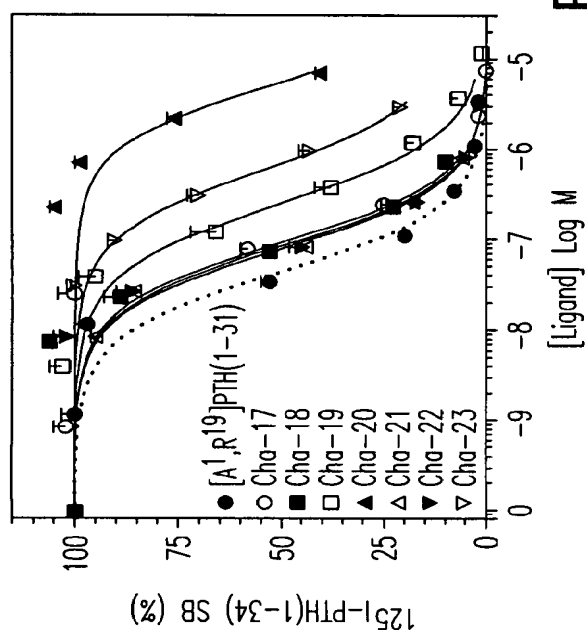


FIG. 3A

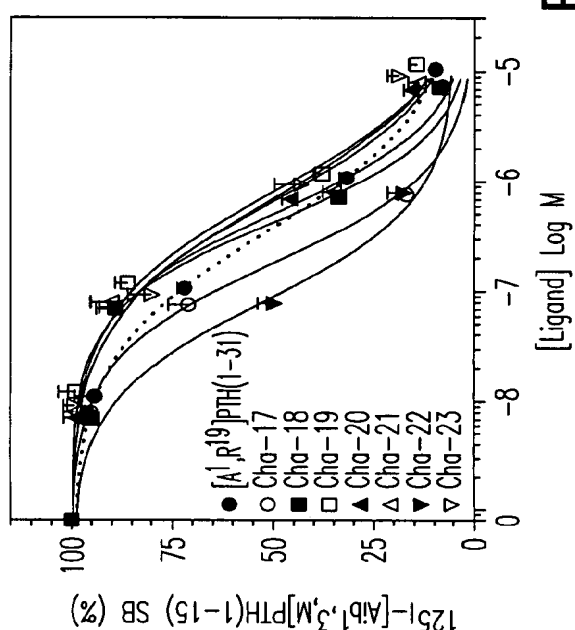


FIG. 3C

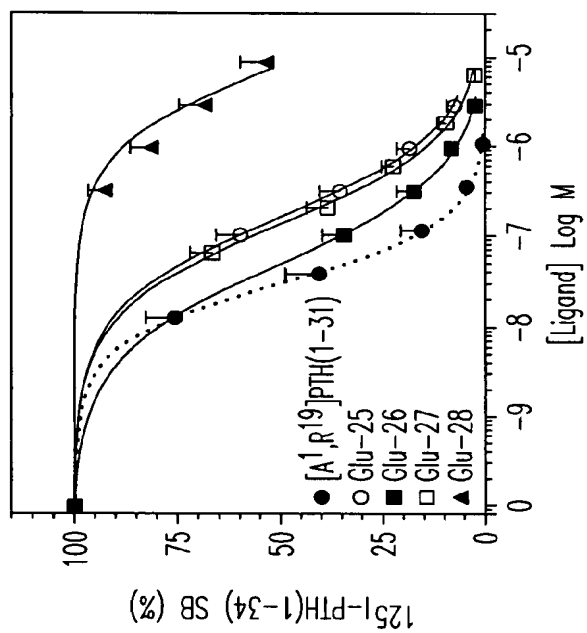


FIG. 4B

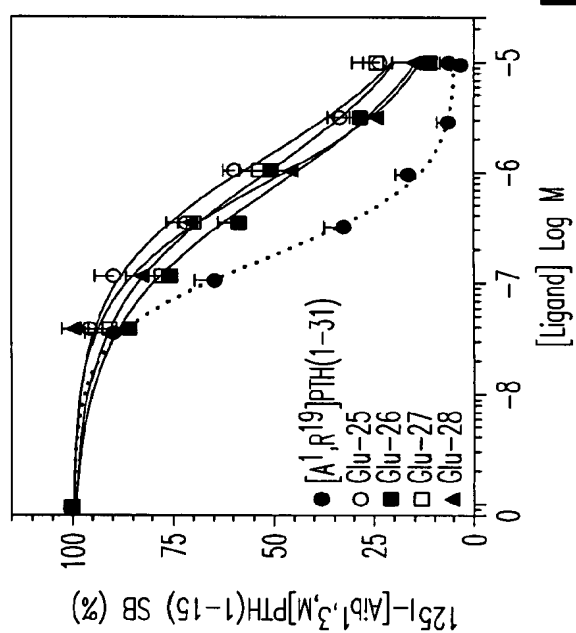


FIG. 4D

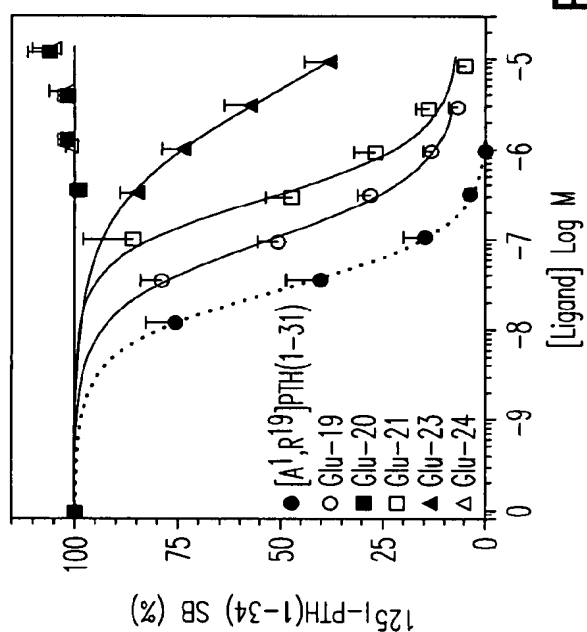


FIG. 4A

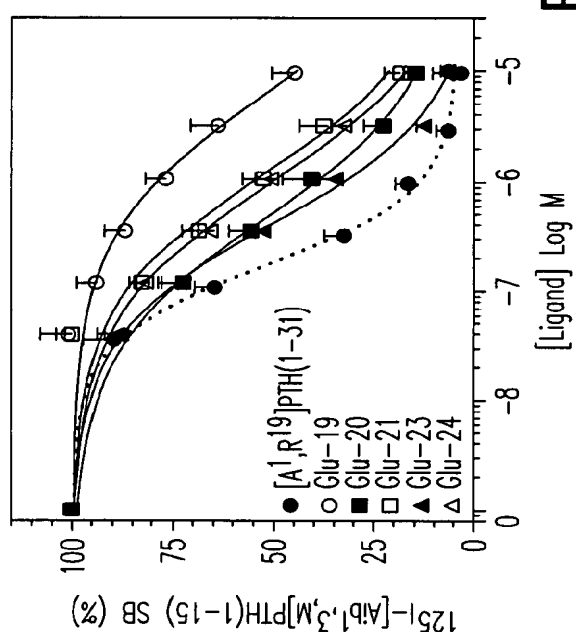


FIG. 4C

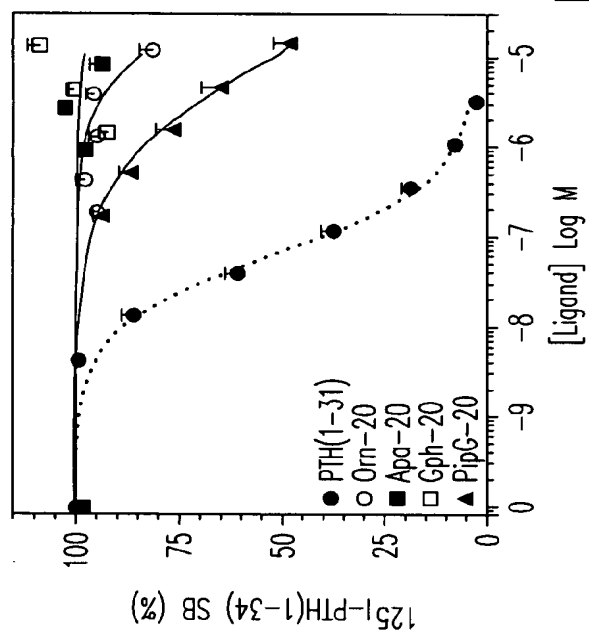


FIG. 5B

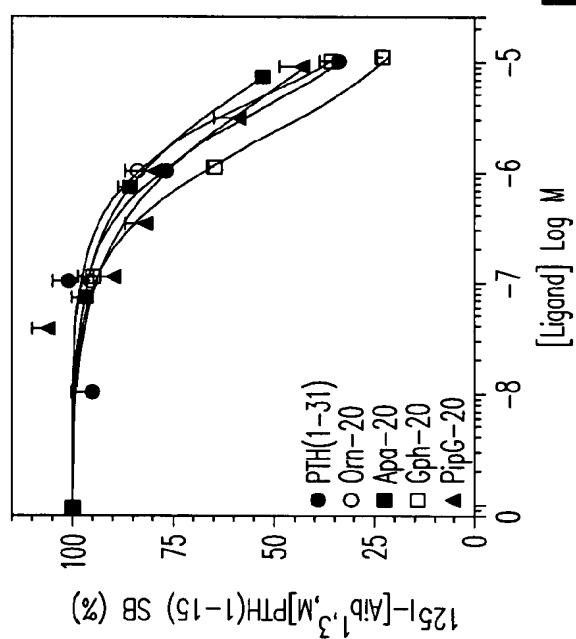


FIG. 5D

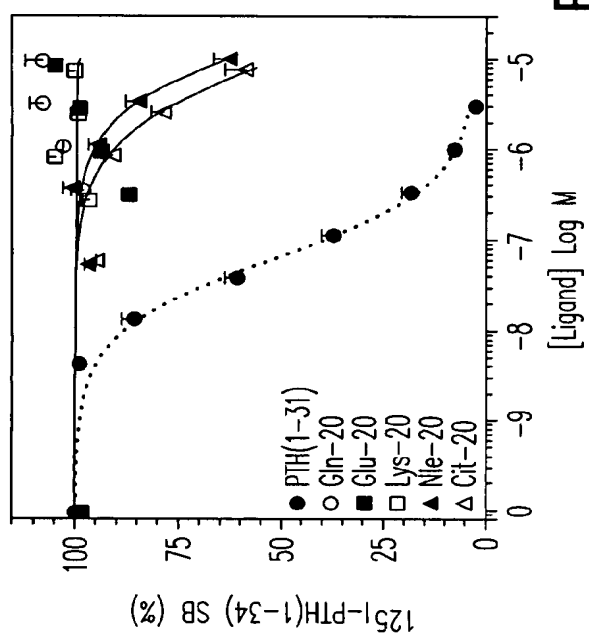


FIG. 5A

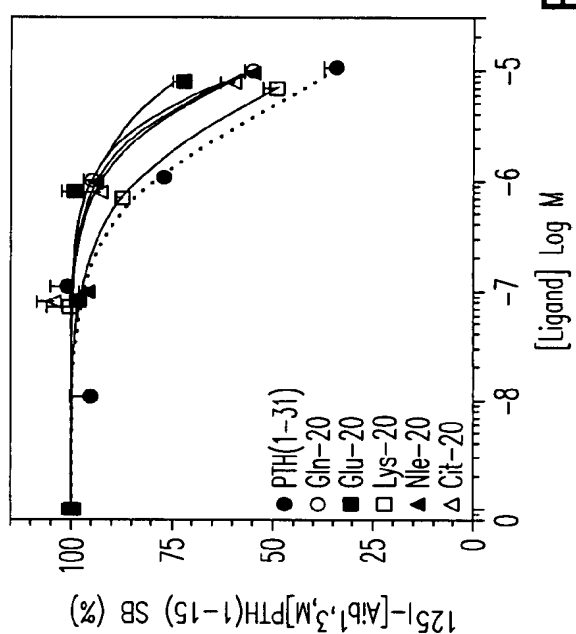


FIG. 5C

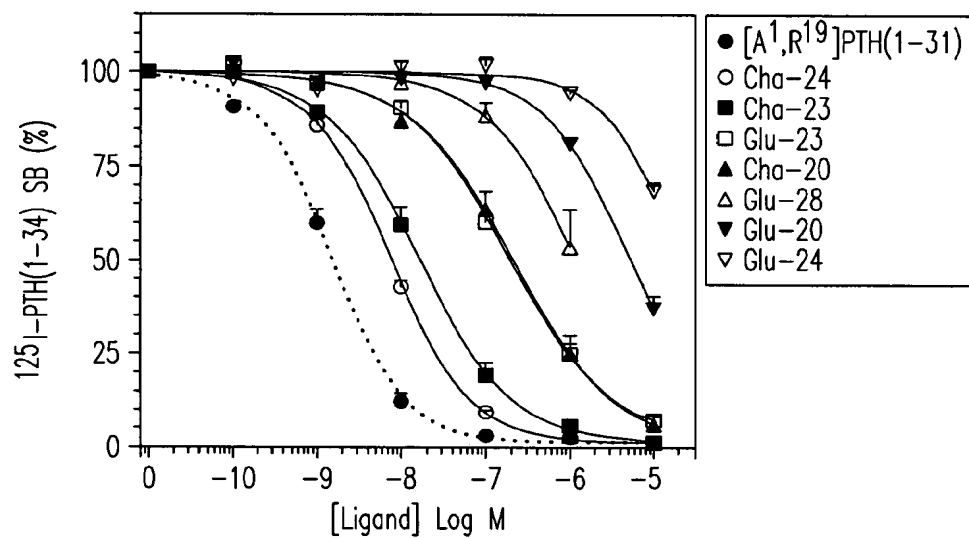


FIG. 6A

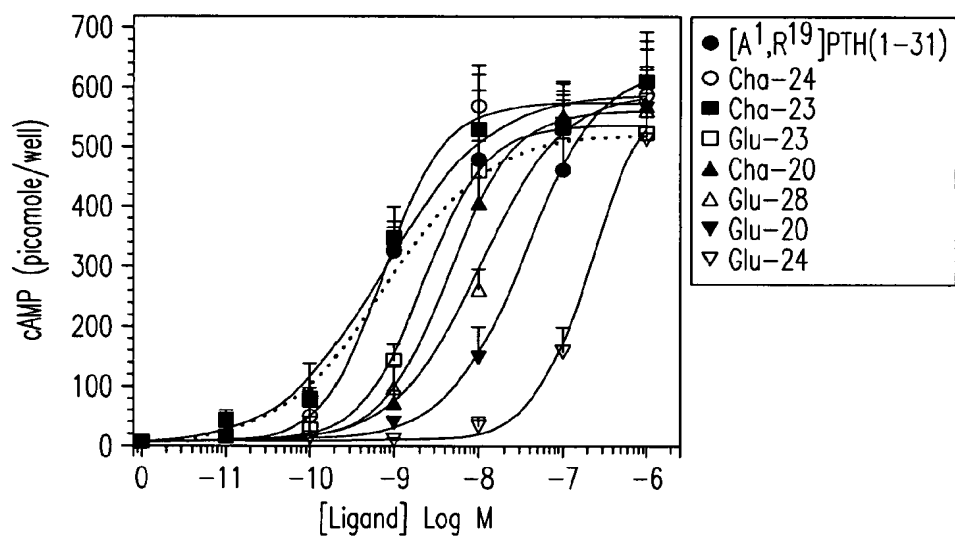


FIG. 6B

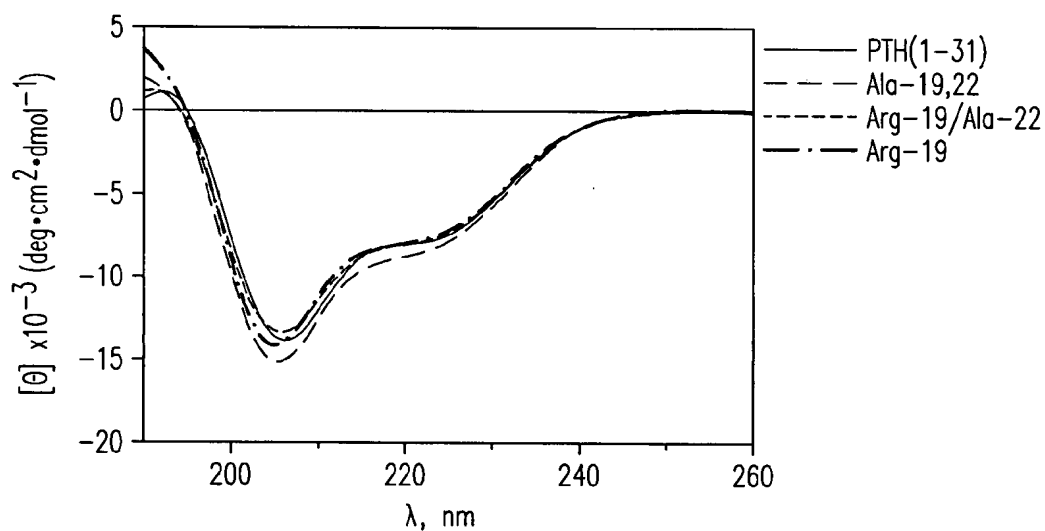


FIG.7A

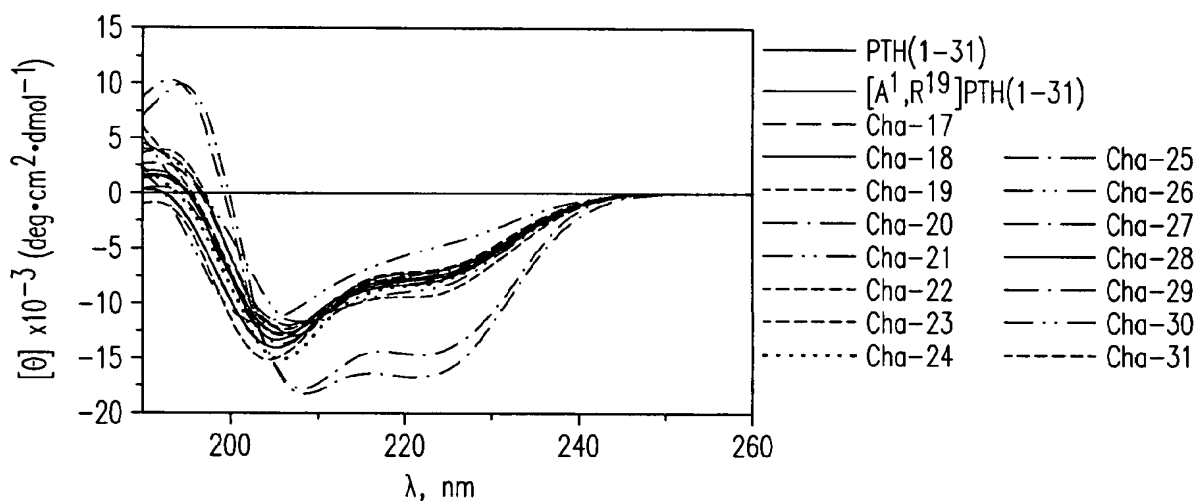


FIG.7B

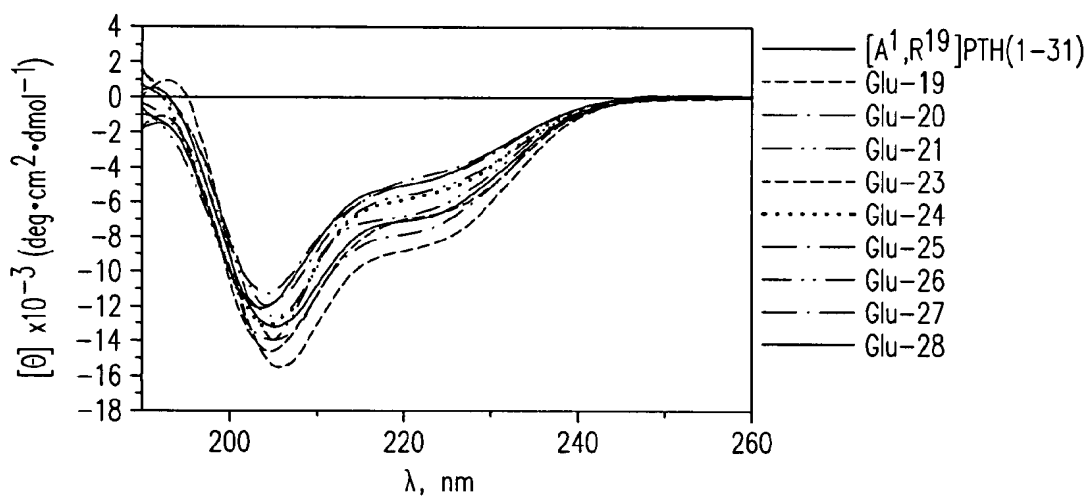


FIG.7C

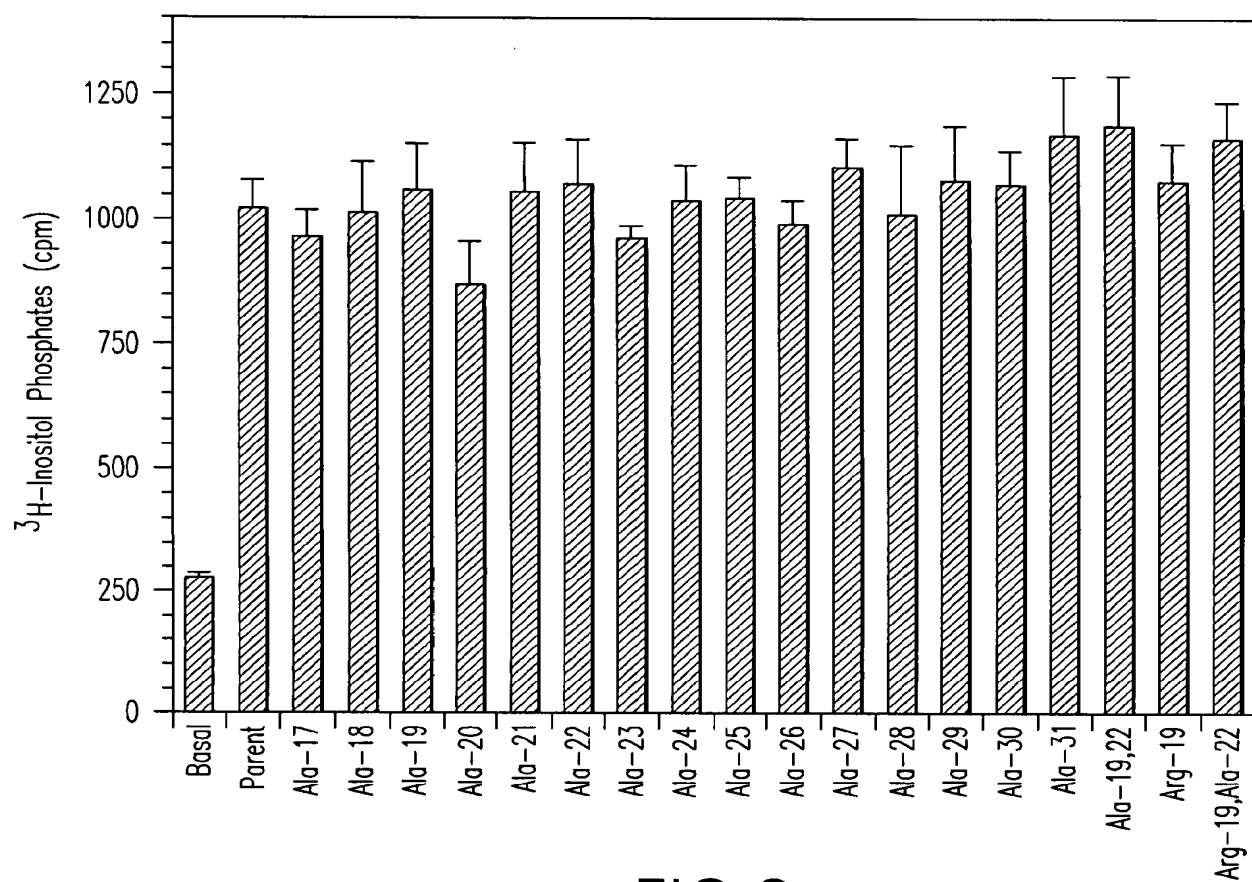


FIG.8